Tamoxifen or Estradiol Limited to the Induction Phase of Nicotine Sensitization Enhances the Expression of Locomotor Sensitization in Ovariectomized and in Intact Female Rats

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Abstract

In both humans and rodents, females are more vulnerable to addiction than males, which has been linked to higher concentrations of the hormone estradiol in females. In rats, nicotine injections produce greater sensitization (neurological changes responsible for drug cravings) in females than in males, which could contribute to sex differences in susceptibility to addiction. The purpose of these experiments was to investigate the effects of estradiol during three nicotine injections (two consecutive days and a third injection nine days later). Results from these experiments indicate that depletion of estradiol via ovariectomy attenuates nicotine sensitization in females, which can be rescued by estradiol injection limited to the induction phase of sensitization. Administration of tamoxifen (antagonist at nuclear estradiol receptors, agonist of the membrane-bound estradiol receptor GPER1) did not alter sensitization in gonadally intact rats, and was sufficient to restore expression of sensitization in ovariectomized females (similar to estradiol). Findings from these experiments indicate that the enhancing effects of estradiol on nicotine sensitization occur during the induction phase, and may be mediated by membrane bound estrogen receptors (e.g., GPER1).

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Abbreviations

4-H-TAM – 4-hydroxy-tamoxifen (metabolite of tamoxifen, interacts with estrogen receptors)

ACh – acetylcholine

D1 – dopamine type 1 receptor

D2 – dopamine type 2 receptor

E2 – 17-ß-estradiol

EB – estradiol-benzoate

GABA – γ-Aminobutyric Acid (primary inhibitory neurotransmitter in the central nervous system)

GPER1 – g-coupled protein estrogen receptor 1

LTP – long-term-potentiation

NAcc – nucleus accumbens

nAChR – nicotinic acetylcholine receptor

NMDA - n-methyl-d-aspirate (Glutamate receptor - critical role in learning and memory & long-term potentiation)

NIC – nicotine

OVX – ovariectomy (or ovariectomized)

S.C. – subcutaneous injection

TAM – tamoxifen

VTA – ventral tegmental area

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General Introduction

The primary goal of research on drugs (including nicotine) is to understand the factors that give rise to the phenomenon of addiction (which can be broadly defined as compulsive drug-seeking behaviour that persists despite known negative consequences; Koob & Volkow, 2010). Over 50% of adults report smoking at least one whole cigarette in their lifetime, but only half of these individuals report ever smoking daily (Stats Canada Smoking report, 2018). Why only a subset of individuals who have been exposed to nicotine progress to develop a pathological addiction is a central question of nicotine research in animals and humans. One factor that is known to contribute to addiction vulnerability is sex: Women transition from occasional to compulsive smoking more rapidly than men (Pogun, Yararbas, Nesil, & Kanit, 2017a), are less responsive to cessation aids such as nicotine patches than men (Perkins & Scott, 2008), and experience more intense nicotine cravings than men during nicotine withdrawal (McClernon, Kozink, & Rose, 2008).

Similarly, female rodents develop compulsive self-administration of nicotine more rapidly than males (Donny et al., 2000; Flores, Pipkin, Uribe, Perez, & Dell, 2016), which makes rats an excellent model to investigate factors that contribute to sex-specific patterns of drug seeking. Importantly, female rodents also exhibit greater sensitization (development of brain modifications that produce drug cravings) during the first few nicotine exposures than do males. Enhanced sensitization in females relative to males may be a key factor in sex differences in addiction liability, as drug cravings are known to promote the transition from casual to

pathological drug seeking (Becker, McClellan, & Reed, 2016; Lynch, 2018; Robinson & Berridge, 1993).

The hormone 17-ß-estradiol regulates reward-motivation in females, and has been shown to enhance drug sensitization across days of testing in female rodents (Hu & Becker, 2003; Martinez, Peterson, Meisel, & Mermelstein, 2014; Zovkic & McCormick, 2019). Brain mechanisms that facilitate enhancement of sensitization by estradiol remain unknown, and could provide targets for sex differences in vulnerability to drug addiction. In this series of experiments, the role of gonadal status and circulating concentrations of estradiol in female rats were investigated during two acute nicotine injections (induction of sensitization), and during a third injection after a delay (expression of sensitization). Because the induction and the expression of sensitization rely on dissociable brain mechanisms, results from the current experiments highlight the time course for the effects of estradiol during the first three exposures to nicotine in female rats.

The problem of nicotine use

Cigarette smoking remains the world-leading cause of preventable death (WHO report 2017), despite global campaigns aimed to increase awareness of the dangers of tobacco smoke. Although most smokers report that they would like to quit, tobacco is addictive, and a high percentage of attempts to abstain from smoking fail (Dappen, Schwartz, & O'Donnell, 1996). Beyond the health consequences that are directly linked to smoking, exposure to the stimulant nicotine is associated with an increased likelihood of experimenting with other illicit drugs, and of developing a substance use disorder (Kandel & Kandel, 2014). Nicotine is also gaining popularity through alternative methods of intake such as vaping (e-cigarette use), which produces serum levels of nicotine that are equal to or greater than those produced by cigarette

smoking (Prossnitz & Hathaway, 2015; Tibboel, Houwer, & Bockstaele, 2015). Although most (62%) e-cigarette users reported they would like to quit, cessation produces nicotine cravings and relapse rates that are similar to cigarette smoking (Rosen & Steinberg, 2019). Consequences of e-cigarette use on future drug-seeking behaviour have not been well characterised, however, smoking e-cigarettes does increase the likelihood of smoking cigarettes a year later (Bell & Keane, 2014). E-cigarette use is also becoming increasingly common in adolescent populations (Perikleous, Steiropoulos, Paraskakis, Constantinidis, & Nena, 2018), who may be especially susceptible to addiction (Crews & Hodge, 2007).

Nicotine may possess unique "gateway" qualities that promote use of other drugs of abuse: infrequent use (as few as 1-2 exposures via cigarette or e-cig), increased predicted odds in engaging in cocaine and marijuana use 4 years later by nearly 200% (Temple, Shorey, Lu 2017). Even occasional nicotine has the potential to heighten the sensitivity of brain reward circuitry, which promotes motivation to seek and use other drug substances (Explanations, Lindsay, & Rainey, 1997; Kandel & Kandel, 2014). People typically report experimenting with cigarette smoking before engaging in illicit substance use (Kandel & Kandel, 2015), which may be related to nicotine's gateway qualities.

In rodents, intermittent nicotine exposure produces long-lasting increases in drug seeking under a variety of experimental circumstances, suggesting a biological basis for nicotine's "gateway" qualities (Kandel & Kandel, 2014). Pre-exposure to nicotine enhances the preference for cocaine-paired environments (conditioned place preference; described later), and nicotine pre-treated rats demonstrated a greater increase in striatal dopamine overflow in response to a cocaine injection that did nicotine-naïve controls (Levine et al., 2014). Nicotine's ability to enhance responses to novel drugs appears to be unique; repeated nicotine injections enhanced the

response to first cocaine, but cocaine pre-treatment had no effect on the first response to nicotine (Kandel & Kandel 2015).

As nicotine use remains prevalent world-wide, understanding the long-term consequences of infrequent exposure may inform models of acquisition and maintenance of drug use in human populations. Establishing modulators of the early changes in the brain produced by intermittent nicotine may help elucidate the mechanisms that produce long-lasting enhancements of reward motivated behaviour. Further, understanding factors that modulate sensitization during intermittent drug exposure may inform models of individual differences in vulnerability to pathological addiction, as sensitization is known to facilitate the development of pathological addiction (Berridge & Robinson, 2016; Robinson & Berridge, 1993).

Historical perspective on substance related disorders and addiction

Brain mechanisms involved in addiction became a subject of interest to neuroscientists because drugs have an extraordinary capacity to drive motivated drug-seeking in human addicts. Historically, drug researchers postulated that the negative symptomology associated with the withdrawal state perpetuated repeated drug use (Dackis & Gold, 1985; Nestler, 1992; Stewart, de Wit, & Eikelboom, 1984). Cessation of chronic drug use produces unpleasant withdrawal symptomology, which can only be ameliorated by more substance. Early models of smoking behaviour in humans thus posited that the aversive withdrawal state promoted conditioned smoking behaviour via negative reinforcement.

Because withdrawal only appears after the discontinuation of chronic use, the withdrawal state cannot explain the initiation of smoking, nor the processes involved in the shift from casual to chronic intake. Further, withdrawal is transient and subsides within days of the cessation of use, yet past drug users remain vulnerable to relapse for years after quitting (Robinson &

Berridge, 1993). Negative withdrawal symptomology may be involved in very selective aspects of addiction (i.e., relapse during early abstinence), however, models of addiction that implicate negative reinforcement as a major driver of addiction cannot account for the initiation of use, or for the long-lasting changes in drug-seeking behaviour (and addiction vulnerability) that endure for years after discontinuation of drug intake.

In response to these conceptual limitations, Booze (1987) put forward a "pleasure seeking" model of drug use, wherein motivational drive to begin and continue using drugs is driven by a desire to experience the hedonic qualities that the drugs offer. In this model, addition is developed and maintained via positive reinforcement.

Contrary to Booze's proposed model, addicts report that the subjective reward value of drugs diminishes as intake escalates (Robinson, & Berridge, 2000). Additionally, if the pleasurable effects of drugs drove the acquisition and maintenance of addiction, the subjective strength of a given drug should be a strong predictor of its abuse liability. Contrary to this qualification, rats will work equally hard for relatively mild psychostimulants (e.g., caffeine, nicotine) and for much stronger mood altering agents (e.g., cocaine, amphetamine), indicating that the hedonic drug qualities alone are not driving motivation to self-administer drugs (Robinson & Berridge, 1993). Pleasurable effects of drugs likely contribute to reinforcement learning during the initiation of drug use, however, a pleasure-seeking model cannot account for the dynamic processes of acquisition and maintenance of addiction.

Negative and positive conditioning effects of drugs in animal models have been proposed under various frameworks (i.e., opponent processes: synergistic effects of hedonic drug qualities and unpleasant withdrawal symptoms). No matter the name, no combination of positive and negative aspects of drug use can fully account for the acquisition of drug addiction.

Current Perspective on Substance Related Disorders and Addiction

The incentive sensitization model of addiction (Robinson & Berridge, 1993) was developed to address the conceptual limitations of opponent processes models of drug seeking. The basis of the incentive sensitization model is that the intensity of drug cravings increases (becomes sensitized) with repeated drug exposures, whereas the subjective hedonic qualities of drugs gradually diminish (a.k.a. "wanting" sensitizes, while drug "liking" decreases; Berridge & Robinson, 2016; Robinson & Berridge, 1993, 2008; Robinson et al., 2000). In accordance with this framework, some smokers report cigarette cravings even during early infrequent use (DiFranza et al., 2007), and more exposure to cigarettes increases reported cravings (DiFranza & Wellman, 2005). Sensitization of the incentive value of drugs (and the corresponding development of drug cravings) is thought to drive the transition from casual and compulsive drug-seeking in this framework. The incentive sensitization model further acknowledges that both positive and negative reinforcement undoubtedly also contribute to the initiation and maintenance of drug use, however, shifts in the incentive salience of drugs drive the long-lasting changes in the brain that underlie pathological addiction (Robinson & Berridge, 1993).

The Diagnostic and Statistical Manual of Mental Disorders (DSM-V) lists 11 criteria for substance use disorder including escalation of intake over repeated uses, substance cravings, development of tolerance, and presence of withdrawal symptomology after cessation of use (for full list see DSM-V). Substance use disorders are categorized as mild (2-3 of 11 criteria met), moderate (4-5 criteria met) or severe (>6 criteria met). Ten substances are recognized in the DSM-V as capable of producing substance use disorder, including tobacco, cocaine, opioids, alcohol and amphetamine. The DSM-V definition of substance use disorder involves the

components that are thought to underlie of the construct of addiction, which can be broadly defined as a loss of control over drug taking (Koob & Volkow, 2010; Eric J Nestler, 2005).

People typically begin by smoking cigarettes occasionally, and smoking trajectories diverge into "smoker" or "non-smoker" during early use (Everett et al., 1999). Some individuals escalate to the point of chronic smoking, eventually producing stable nicotine concentrations across the day, whereas others never progress beyond infrequent smoking. In a 4-year longitudinal prospective study commencing in the 6th grade, researchers found that about half the individuals who tried smoking lost autonomy (sense of control) over their tobacco use (DiFranza & Wellman, 2007). The most susceptible youths (10% of inhalers), reported the loss of autonomy over smoking within 2 days of their first cigarette, and 25% reported the loss of autonomy within 30 days. Factors that predict the occurrence and speed of loss of autonomy after first use remain of great interest to drug researchers, as they may facilitate the transition from mild to severe substance use disorder, and may also promote experimentation with other drugs.

The Mesolimbic Reward Pathway and Drugs of Abuse

The mesolimbic reward pathway is primarily comprised of dopaminergic cell bodies within the ventral tegmental area (VTA) that project axon terminals to the nucleus accumbens (NAcc; See fig 1A), and is a key brain target for drugs of abuse. The mesolimbic circuit is highly conserved across mammals, birds, fish, and insects, because the ability to learn and identify rewarding stimuli putatively aids survival in a natural environment (Scaplen & Kaun, 2016). The presence of natural rewards (e.g., food, sex), initiates increased phasic firing patterns and dopamine (primary neurotransmitter of the reward pathway) release from the VTA to the NAcc, which initiates approach-motivated behaviour via disinhibition of the prefrontal cortex (see Fig. 1). In nature, motivated reward-seeking behaviour is involved in driving food- and sex-seeking,

which ultimately promotes survival and reproduction, enhancing evolutionary fitness. Dopamine signalling also enhances memory acquisition, rapidly pairing perceived reward with environmental cues via associative conditioning. An enhanced memory for rewarding stimuli (and associated environmental cues) may promote the repetition of behaviours that previously led to natural rewards.

Although addictive drugs have various mechanisms of action in the brain, all drugs that have the potential to produce addiction (e.g., nicotine, caffeine, opiates, alcohol, amphetamines) cause increases in dopamine release from VTA terminals to NAcc neurons when administered acutely (Nestler, 2005). This common characteristic of addictive drugs implicates the mesolimbic dopaminergic system as a primary candidate for investigates of drug-induced alterations in reward motivation (rev in De Kloet et al. 2015).

Within the NAcc, two dopamine receptor subtypes (low affinity dopamine type 1 (D1) receptors and high affinity dopamine type 2 (D2) receptors) are involved in modulating reward and aversion, respectively. Under baseline conditions, when dopamine concentrations in the NAcc are low, more binding occurs at D2 receptor sites than at D1. When dopamine spikes after reward presentation, proportionally more dopamine binding shifts to D1, as the majority of D2 sites become saturated. NAcc D1 receptors are found on γ-Aminobutyric Acid (GABA)-ergic medium spiny neurons, which project axons to GABAergic cell bodies in the substantia nigra and VTA. This downstream subpopulation of GABAergic cells has both cortical and thalamic targets (primarily motor-related regions) under tonic inhibition at baseline (see Fig. 1A). When drugs are present in the system (and dopamine output to the NAcc increases), binding at D1 receptors increases GABA output from NAcc medium spiny neurons, which then inhibit downstream VTA GABA cells, which in turn release their targets from tonic inhibition

(disinhibition), ultimately producing increases in approach motivated behaviour (see Fig. 1C; rev. in Nakanishi et al., 2018). Selective ablation of dopamine cells in the VTA or the NAcc by 6-hydroxy-dopamine lesions diminished drug-seeking behaviour (Roberts & Koob, 1982), and D1-receptor knockout mice also do not self-administer drugs of abuse (Caine et al., 2007). Taken together, these findings implicate D1-mediated signalling in the NAcc as a necessary component in the initiation of drug-seeking behaviour, and by extension, a key modulator of the development of the addiction.

Mechanisms of nicotine action

Nicotine produces actions within the central nervous systems of both humans and rodents that are sufficient to give rise to pathological addiction (Markou, 2008). People report high addiction liability after repeated nicotine use, experience unpleasant withdrawal symptomology during cessation of chronic intake, and relapse on average 30 times before successfully quitting smoking (Pogun, Yararbas, Nesil, & Kanit, 2017b).

Nicotine's primary target within the central nervous system the nicotinic acetylcholine receptor (nAChR; pentametric non selective cation channels found on post-synaptic membranes). Nicotine mimics the endogenous ligand acetylcholine's actions at nAChRs; binding produces a conformational change among the 5 subunits, which shift their positions to open a central pore through which ions can enter the cell. As a non-selective cation channel, the nAChR allows positively charged ions (sodium, calcium etc.) to transverse the pore when open, increasing the resting membrane potential and cellular excitability of the neuron.

Cholinergic circuitry modulates the mesolimbic dopamine system via several indirect mechanisms: Acetylcholine signalling from hindbrain nuclei (primarily from the mesopontine tegmentum; comprised of the lateral dorsal tegmental nucleus and the pedunculopontine

tegmental nucleus, Maskos, 2008) activates the dopamine neurons in the VTA, a direct cholinergic projection extends from the VTA to the NAcc, and GABA interneurons express nAChRs within the NAcc to modulate feedback to the VTA (De Kloet, Mansvelder, & De Vries, 2015). The primary mechanism by which nicotine enhances reward signalling is by binding to nAChRs within the VTA, promoting release of dopamine to the NAcc (rev. in De Kloet et al. 2015, see fig. 1).

Models of drug seeking in rodent studies

Rats will voluntarily self-administer nicotine when given the opportunity (Balfour, Benwell, Birrell, Kelly, & Al-Aloul, 1998), and demonstrate evidence of withdrawal symptomology after chronic nicotine is discontinued (Wilmouth & Spear, 2006), which makes rats an excellent model for investigations of nicotine's effects on brain and behaviour. There are three classical models used to investigate reward motivation for drugs in rodents, described below.

Self-administration. Self-administration paradigms typically involve isolating rats in operant chambers, tethered to a drug source that delivers infusions either into circulation (e.g., via jugular catheter) or directly to the brain (e.g., via stereotaxic cannulation). Rats will self-administer the same substances that are capable of producing addiction in humans (i.e. opioids, psychostimulants, alcohol), indicating that the mechanisms responsible for addiction may be conserved. Additionally, rats escalate intake across days of testing when given free access to drugs, which is representative of the typical patterns of onset of drug use in human populations (Goodwin, Hiranita, & Paule, 2015), suggesting that self-administration paradigms offer a high criterion validity to the onset of drug use in people. Some (but not all) rodents rapidly progress to compulsive self-administration, promoting tolerance to drug effects and withdrawal symptoms

after access is removed. The propensity to self-administer drugs of abuse makes rodents an ideal model for investigation of mechanisms of action, and consequences of exposure to drugs on brain functioning and future drug-seeking behaviour.

One way to measure the willingness to self-administer is to count the number of infusions earned under a fixed ratio schedule, where a set number of bar presses results in an infusion (e.g., fixed ratio 1 schedule = every bar press earns an infusion). A threshold for compulsive or "binge-like" behaviour is identified (i.e. 20 infusions / day), and researchers can assess the rate at which intake escalated to meet the operationalized threshold. Another commonly used method to assess self-administration is to use a progressive ratio schedule, wherein the number of bar presses required to produce an infusion is doubled after each successful dose (i.e. one, two, four, eight, sixteen bar presses to obtain an infusion). Under a progressive ratio schedule, the "break-point" is defined as the highest number of presses that earned an infusion, and shifts in the break point are an indication of motivation to work for the infusions.

A criticism of self-administration paradigms is that not all rats obtain the same number of infusions during training, and as such doses of drug exposure are not consistent across all subjects. Nevertheless, information from self-administration paradigms may be especially informative about the motivation for drug infusions, as animals freely choose whether (and how much) to self-administer.

Conditioned place preference (CPP). During place preference training, rats are repeatedly injected with a drug and placed in a test apparatus with distinct cues (e.g., wire mesh floor and horizontally striped walls). In separate test sessions, the same rats are repeatedly injected with saline and exposed to a test environment with equally distict but different cues (e.g., smooth floor and polka dotted walls). Reward value of a given substance is evaluated by

allowing rodents to choose between the drug-paired and the saline-paired environment in a drug free state (Fernandez, 2015). More time spent in the context that has been paired with drugs indicates that the rat remembers the conditioning session, and that they found the experience of drug effects rewarding. One strength on conditioned place preference training is that animals are tested in a drug-free state, however, considerable variation in the protocols used for training and data analysis complicate interpretations of results from studies that involve CPP paradigms.

Drug sensitization. When repeatedly administered, all addictive drugs (including nicotine) produce stepwise increases in locomotor activity (Booze, Welch & Wood, 1999, Domino, 2001, Miller, Bardo & Wilkins, 2001, Goutier, Kloeze & McCreary, 2016), and NAcc dopamine overflow (Balfour, Benwell, Birrell et al. 1998, Cadoni & Chiara, 2000; Domino 2001; Shim 2001) (i.e., repeated injections produce escalating hyperlocomotion and extra-synaptic dopamine in the NAcc). Heightened sensitivity of the dopaminergic reward circuit is thought to increase the incentive value of nicotine, which leads to enhanced motivational drive and promotes drug seeking (i.e., craving or "wanting"; Robinson & Berridge, 1993, 1998, 2008, Berridge & Robinson, 2016). A persistent and incremental increase in dopamine overflow and locomotor activity are visible either when nicotine is injected systemically (Benwell & Balfour, 1992) or delivered stereotaxically to the VTA (Stolerman, 1990). Sensitization represents a pre-clinical model for the development of insatiable drug craving, whereby stepwise increases in DA overflow in response to drugs drive the increases in motivated behaviour for continued drug seeking (Chiara, 1995, Robinson & Berridge, 1993, 1998, 2008, 2016).

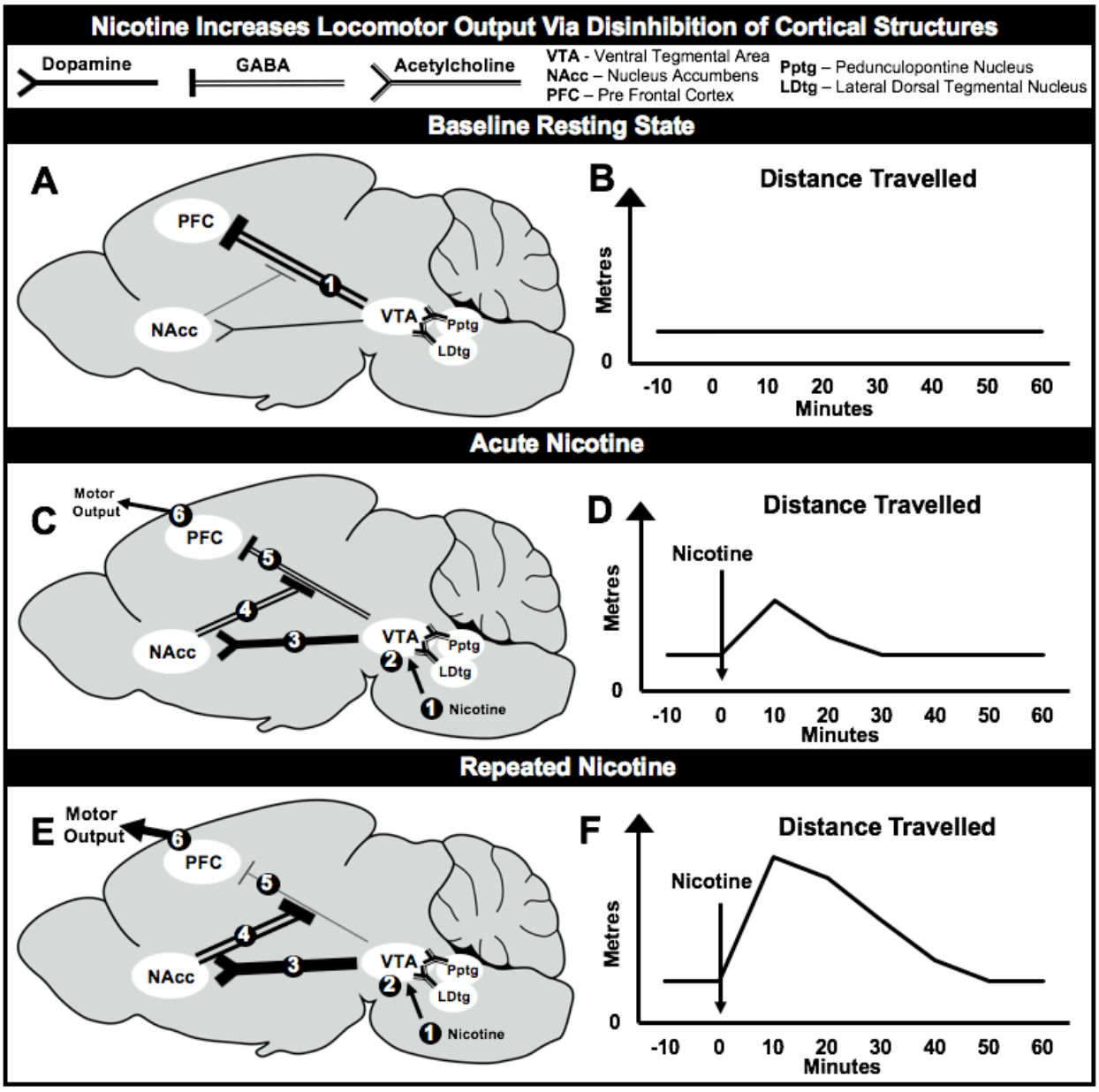

Figure 1. Simplified diagram of brain pathways involved in motor disinhibition by nicotine, depicted on a sagittal view of a rat brain. (A) At rest, GABAergic VTA neurons hold cortical targets under tonic inhibition, which results in steady low levels of locomotor activity (B). (C) When nicotine (1) is present in the system, it mimics the actions of acetylcholine at VTA nAChRs, producing increased cellular excitability in the VTA (2). More excitable VTA dopaminergic neurons increase dopamine release to the NAcc (3). When the NAcc is flooded

with dopamine, GABAergic interneurons in the NAcc inhibit VTA GABAergic cells (4), resulting in disinhibition of cortical targets (5). Releasing cortical targets from tonic inhibition results in observable increases in ambulatory movements (e.g., locomotor activity; depicted in (D)). (E) When nicotine is repeatedly administered, the mesolimbic dopamine response becomes "sensitized": when nicotine (1) is present in the system, actions at VTA nAChRs produce a proportionally larger release of dopamine (3), which results in increased inhibition of the GABA neurons (4) that inhibit the cortex (5). Increased disinhibition in sensitized rats (relative to drug naïve control; B) produces greater increases in locomotor activity after injection (F).

During sensitization testing, rodents are repeatedly administered drugs on a series of test dates (rather than continuous infusions), and tested for NAcc dopamine overflow and / or locomotor activity in the hour after injection. Administering repeated nicotine injections is representative of the onset of smoking in people, which typically begins with occasional use (DiFranza & Wellman, 2007). Sensitization can be measured by within-individual changes across days of testing, or as a between-group difference to a "challenge" injection (administered after a delay), whereby drug-pretreated animals are compared with saline-pretreated control subjects. Each analysis strategy presents strengths and weaknesses, and the statistical approach used to analyze sensitization data should be chosen carefully based upon the specific research questions.

Although locomotor activity and dopamine release both sensitize across acute administrations of nicotine, they do not occur simultaneously after nicotine administration. Locomotor activity increases almost immediately, and peaks 10-15 minutes after systemic nicotine injection (Matta et al. 2006), around the time nicotine begins to reach the brain. The

onset of increased dopamine release in the NAcc occurs about 20 minutes after injection, and peaks around 60 minutes post-nicotine (Shim et al. 2001). Parallel responses have been reported from human smokers: Participants rated the subjective effects of nicotine highest while they were smoking, and these subjective ratings quickly declined after the end of the smoking session. Peak nicotine blood levels, as well as the smoking-induced release of adrenocorticotropin releasing hormone and cortisol, occurred after the subjective reports had declined (Mello, 2010).

An increased motivational drive to seek nicotine (and possibly other drugs as well) caused by the sensitization of incentive salience is thought to be a key factor in the development of addiction, as the neuroadaptations produced by sensitization are sufficient to be considered pathological in nature (Berridge, 2017; Markou, 2008). Behavioural correlates of a previous nicotine exposure (expression of sensitization) endured for up to seven months of nicotine abstinence in rats (Morud, Adermark, Perez-alcazar, Ericson, & Söderpalm, 2015), suggesting that the neuroadaptations that underlie sensitization are long-lasting enough to likely represent permanent changes to the brain (rev. in Robinson & Berridge, 1993). Rats that show a greater sensitization to the incentive salience of a food reward self-administer more cocaine and show a greater preference for a cocaine-paired environment than do those that do not show sensitization of incentive salience to food reward (Beckmann, Marusich, Gipson, & Bardo, 2011), implicating individual differences in sensitization as a predictor of drug-seeking.

One model that has been used to investigate individual variability in susceptibility to drugs is the high / low responder model, where rats are selectively bred for high or low locomotor response to a novel environment (likened to the human trait of sensation seeking; Piazza & Moa, 1996). High responders will work harder for infusions of nicotine than will low responders, and high-responders also self-administer more nicotine when given free access

(Suto, Austin, & Vezina, 2001). During a series of acute injections of nicotine, high responders demonstrated sensitization of locomotor activity sooner than low responders, and high responders travelled longer distances when injected with nicotine again after an eight day delay (Pawlak & Schwarting, 2005). In vivo microdialysis studies indicated that an acute injection of nicotine produces increased dopamine release in the NAcc of high responders, and a decreased dopamine release relative to baseline in low responders (Siciliano, McIntosh, Jones, & Ferris, 2017). For cocaine, female high responders self-administered more infusions than did female low responders, and male high and low responders did not differ in the number of infusions they self-administered (Davis, Clinton, Akil, & Becker, 2008); male and female high and low responders have not yet been investigated for their responses to nicotine. It remains unknown whether greater sensitization in high responders contributes to their greater propensity to self-administer nicotine and other drugs as compared to low responders. Nevertheless, taken together, these findings implicate the sensitization of the mesolimbic dopamine response to nicotine as a possible correlate of development of self-administration behaviour in rats.

Induction and expression of drug sensitization

Continuous nicotine administered via osmotic mini-pump does not produce a sensitization of locomotor activity or a release of dopamine in the NAcc (Baker et al., 2013; Benwell, Balfour, & Birrell, 1995). However, rats do show sensitization to an acute nicotine injection 24 hours after the removal of the mini-pumps (Faraday, Donoghue, & Grunberg, 2003), suggesting that sensitization requires both the presence of the drug for some time, and a drug free period after exposure (Vezina, McGehee, & Green, 2007). For this reason, sensitization is typically separated into two anatomically and functionally distinct phases: induction and expression (DiFranza & Wellman, 2005; Vanderschuren & Kalivas, 2000). The induction phase

involves the responses to the first exposure (or exposures) and the development of the neurological changes that underlie sensitization. The expression phase of sensitization refers to the behavioural response to a nicotine challenge administered after a longer period of abstinence (rev in Vezina et al., 2007).

Independent mechanisms for the induction and the expression of behavioural sensitization have been elucidated using pharmacological approaches. For example, antagonism of n-methyl-d-aspartate receptors (a glutamatergic receptor known to play a key role in learning and memory; NMDA) blocks the induction (but not the expression) of sensitization, indicating that acquisition of sensitization depends on NMDAr-mediated signalling whereas expression does not. Further, blocking nAChRs via stereotaxic injection of the nAChR antagonist mecamylamine blocked the induction of sensitization when infused in the VTA (but not when into the NAcc), suggesting that VTA nAChRs are necessary to acquire sensitization and that NAcc nAChRs are not (Baker et al., 2013). On the other hand, antagonism of cannabinoid type-1 receptors attenuated the expression, but did not alter the induction, of nicotine-induced locomotor sensitization (Kelsey & Calabro, 2008), indicating that cannabinoid signalling is selectively involved in the expression of sensitization. Although these phase-specific mechanisms are certainly not exhaustive, they indicate that there are distinct neural substrates for the induction and the expression of locomotor sensitization to nicotine.

Despite evidence of distinct anatomical and functional correlates of the induction and the expression of nicotine-induced sensitization, most injection protocols involve repeated nicotine and testing for locomotor activity with equal delays between injections (i.e. every day or every other day;Booze et al., 1999; Fennell, Pitts, Sexton, & Ferris, 2019; Goutier, Kloeze, & McCreary, 2016; Harrod et al., 2004; Illenberger, Mactutus, Booze, & Harrod, 2018; Kanýt,

Stolerman, Chandler, Saigusa, & Pögün, 1999; McCormick, Robarts, Gleason, & Kelsey, 2004). Repeatedly testing rats with equal withdrawal periods between test dates does not allow for separation of the induction and expression of sensitization.

Sex differences in smoking behaviour in humans

Women smokers self-report a quicker transition from casual to compulsive nicotine use than do men (i.e., the acquisition of addiction) (Pogun et al., 2017b). Women also report more difficulty with the cessation process than do men (Wetter et al., 1999), and are more likely than are men to succumb to relapse after a period of abstinence (rev. in Pogun 2017a). Women also report that they find nicotine's effects more pleasurable than do men, and demonstrate more negative affective symptomology than do men during withdrawal (Hogle & Curtin, 2006). Sex differences in the development of addiction occur primarily during the experimentation phase, where use transitions from casual to compulsive (e.g., for heroin, Hser, Anglin, & Booth, 1987), indicating that sex-specific patterns of sensitization may be a good mechanistic candidate for understanding the mechanisms that underlie sex differences in addiction vulnerability. The greater vulnerability to addiction in women relative to men is also observed for illicit drug use (Bobzean et al. 2014, Becker 2017). One hypothesis as to why females are more vulnerable to addiction than are males is that the higher levels of estradiol in females promote addiction (Lynch, 2009), however, the specific mechanisms by which estradiol enhances addiction are unknown.

Reward-motivation across the estrous cycle in females rodents

Unlike humans, female rodents undergo a 4-5 day estrous cycle, during which circulating levels of the primary ovarian hormones (estradiol and progesterone) fluctuate. Female rats reject sexual advances during non-fertile times (i.e., diestrus / metestrus phases of the 4-5 day cycle),

and only engage in proceptive and receptive sex behaviours around the time of ovulation; termed "behavioural estrus" (Hamid, Abu, & Zakaria, 2013). Displays of other reward-related behaviours such as wheel-running and intra-cranial self-stimulation increase during the same interval that rats would be receptive to sex (peaks during the night between proestrus and estrus; Steiner et al., 1981). Dopaminergic agonists also produce the greatest behavioural effects during the time that corresponds to behavioural estrus in female rats (Steiner, Katz, & Carroll, 1980). Shifts in reward-motivated behaviour mediated by fluctuations of ovarian hormones (primarily estradiol) may adjust sex-seeking behaviour as a function of fertility during each phase to account for risk / reward evaluation of the likelihood of conception. Taken together, these findings implicate estradiol as an important modulator of reward motivation in females. It is possible that the neural circuitry involved in modulating sex-motivated behaviour during behavioural estrus may also modulate drug seeking in females.

Inherent challenges in studying effects of gonadal hormones on reward motivated behaviour

Studying the modulatory effects of gonadal hormones on drug-seeking behaviour in animal models presents a unique challenge. Female rodents undergo a 4-5 day estrous cycle, in which hormonal shifts can be roughly estimated by measuring the quality and quantity of epithelial cells in vaginal lavage samples. One challenge with investigations of cycle phase is identifying the direct effects of ovarian hormones on reward-seeking behaviour, as estradiol and progesterone both rise and fall across the estrous cycle. The shifting levels of two hormones that have both independent and interactive effects on brain systems limit inferences about the effects of either hormone on its own in experiments involving gonadally intact females.

To circumvent this problem, many researchers instead use models in which circulating concentrations of ovarian hormones can be controlled. Removal of the ovaries via ovariectomy (OVX) is sufficient to deplete endogenous concentrations of the estradiol and progesterone from the blood circulation, which allows for the exogenous replacement of one or both hormones. One common method to administer exogenous ovarian hormones is to implant pellets packed with estradiol and / or progesterone below the skin at the time of OVX, which release hormones slowly across a long period of time. Implants have retained popularity because they are effective at producing relatively consistent estradiol concentrations in serum, simple to implant, and do not require administration by injections (which can be stressful to rats). After OVX, the density of dendritic spines in the hippocampus and NAcc decreased steadily for approximately 7 days before stabilizing, and exogenous estradiol after OVX prevented the loss (Woolley & McEwen, 1993). As such, relevant receptor targets as well as the structural morphology of the brain may be different in estradiol implanted than in OVX + empty implant rats at the onset of behavioural testing, as animal care guidelines typically require a 5-day recovery period at minimum. Further, slow-release implants maintain relatively consistent circulating estradiol concentrations for the duration of the experiment, which is not representative of the cyclic patterns of endogenous estradiol, which rise and fall across the estrous cycle.

Another way to administer hormones in an OVX model is to administer estradiol and / or progesterone at specific time points via systemic or stereotaxic injection. Rats are typically given a minimum of 5 days to recover from OVX surgery before any experiment, thus all individuals would undergo the morphological changes associated with depletion of ovarian hormones described above (see Woolley & McEwen, 1993). Studying the effects of ovarian hormones in a depleted system may not be representative of the endogenous actions of estradiol in a gonadally

intact female. This caveat should be considered when interpreting results from OVX models, however, the goal of the OVX / selective replacement design is not intended to mimic the hormone fluctuations that occur across the estrous cycle. Rather, this approach allows for tightly controlled investigations of the independent and interactive effects of ovarian hormones on measures of brain and behaviour.

Estrogen receptors in the brain

The classical receptors for estradiol (ERα and ERß) reside in the cytoplasm of the cell when unbound. Estradiol can transverse cell membranes to interact with its receptors, which facilitates receptor dimerization wherein two estradiol-bound receptors join together and form a complex that can translocate to the nuclear compartment of the cell. Within the nucleus, the ER complex interacts with estrogen-response-binding elements on DNA strands, and also has the potential to alter gene expression through epigenetic mechanisms (Hamilton, Hewitt, Arao, & Korach, 2017).

Evidence for the possibility of membrane-bound estrogen receptors began to accumulate as early as the 1970's, but at that time, many researchers have speculated that they were simply classical ER units embedded in the cell membrane (Brailoiu et al., 2006). In the late 1990's, four independent research teams using different experimental approaches all identified the same 7-trans membrane-bound estrogen receptor, named the g-coupled protein estrogen receptor 1 (GPER1; previously known as the orphan g-coupled protein receptor GPR 30; Gaudet, Cheng, Christensen, & Filardo, 2015). GPER1 initiates intracellular cascades that ultimately upregulate phosphorylation of the extracellularly regulated kinase (ERK) 1 / 2, which in turn activates protein kinase A. Upregulation of ERK 1 / 2 and protein kinase A phosphorylation may promote

processes such as long term potentiation (strengthening or formation of new synapses) in the brain (Fernandez et al., 2008)

Synthetic estrogenic compounds used in laboratory research

One way to investigate the effects of estradiol on brain and behaviour is to administer the most abundant endogenous estrogen (17-ß-estradiol) via acute injections to OVX females. This approach allows for rigorous control of the amount and frequency of estradiol exposure. 17-ß-estradiol is cleared from circulation quickly, and is undetectable 6 hours after a physiologically relevant acute injection (Layendecker et al. 1975). To extend the duration of action of exogenously administered estradiol, the pro-drug estradiol-benzoate was developed. Estradiol-benzoate has low affinity at estrogen receptors (1% that of 17-ß-estradiol), but undergoes bio-transformation to produce 17-ß-estradiol. Because transformation of estradiol-benzoate to estradiol requires additional time, serum levels of 17-ß-estradiol remain elevated for several hours after acute estradiol-benzoate injection, as compared to administration of 17-ß-estradiol (Layendecker et al. 1975).

One synthetic estrogen receptor modulator that has been available since the 1960's is tamoxifen, which is an antagonist of the classical estrogen receptors ERα and ERß. Tamoxifen itself does not bind to estrogen receptors, however, one notable bio-metabolite (4-hydroxy-tamoxifen; 4-H-TAM) binds to the dimerization port on ERα / ERß, which prevents receptors from creating dimers, thus forcing bound units to remain in the cytosol (Prossnitz, Oprea, Sklar, & Arterburn, 2009). Because 4-H-TAM binding at estrogen receptors prevents translocation to the nucleus, classical genomic effects are effectively blocked (Jabar et al 2006). 4-H-TAM also acts as an agonist at the membrane-bound estrogen receptor GPER1, without showing preference for any type of estrogen receptor (Prossnitz et al. 2009). Because tamoxifen activates GPER1 and

antagonizes the classical estrogen receptors, it offers an interesting tool to explore whether an established effect of estradiol depends on the canonical receptor subunits or involves actions at GPER1. Other synthetic drugs are available that have actions specific to one receptor sub type, however, I am limiting the discussion to tamoxifen here because it is the drug used in some of the present experiments.

Estradiol and drug-seeking in female rodents

When the number of lever presses required to obtain a cocaine infusion is increased progressively, rats in the estrus phase of the cycle will work 2x more than those in all other phases, suggesting an increased motivation for cocaine reward during estrus (Roberts, Bennett, & Vickers, 1989). Females in the estrus phase also showed significantly more striatal dopamine release than did females in the diestrus phase when exposed to equal doses of amphetamine, measured both in-vivo by microdialysis and in vitro using striatal tissue (Becker & Cha, 1989).

Depletion of circulating ovarian hormones in females via OVX diminishes the sex differences in cocaine self-administration (Hu, Crombag, Robinson, & Becker, 2004), indicating that ovarian hormones may promote self-administration in females. OVX females acquired cocaine self-administration at the same speed as males, and estradiol (but not progesterone) administration enhanced speed of acquisition of self-administration in females only (Hu & Becker, 2008; Jackson, Robinson, & Becker, 2006). Further, estradiol (but not progesterone) enhanced dopamine overflow in the NAcc in response to first amphetamine in female subjects in vivo (Becker & Rudick, 1999), and systemic estradiol injection enhanced amphetamine-induced dopamine release in a striatal slice preparation after estradiol was added to the medium (Becker & Beer, 1986). Administration of estradiol to the dorsal striatum through implanted cannulae was also sufficient to enhance NAcc dopamine overflow to amphetamine injection later in vivo

(Cummings, Jagannathan, Jackson, & Becker, 2014; Shams, Sanio, Quinlan, & Brake, 2016). The enhancing effects of estradiol on brain responses to psychostimulants are also sexually dimorphic; estradiol injections in male rats did not augment dopamine release after cocaine injection (Bazzett & Becker, 1994) as they did in females.

A recent report from our lab found that estradiol-benzoate administered 30 minutes before an amphetamine challenge enhanced the expression of locomotor sensitization in OVX female rats (and had no effect on locomotor activity during the induction phase; Zovkic & McCormick, 2019). Another report found that injection of estradiol 30 minutes before amphetamine was also sufficient to enhance NAcc dopamine overflow (Bazzett & Becker, 1994), further indicating that estradiol's enhancing actions may be mediated by a rapid mechanism (i.e., membrane bound g-protein coupled estradiol receptors e.g., GPER1), as 30 minute pre-treatment is unlikely to allow for transcriptional activity of ERα / ERß. Taken together these reports indicate that estradiol is an important modulator of drug seeking behaviours, and of drug-induced dopamine release, in female (and not in male) rats.

The role of ovarian hormones in nicotine-seeking in rats

In accordance with investigations using illicit psychostimulants, self-administration paradigms involving nicotine indicate that female rats acquire self-administration more rapidly than do males, but do not consume more than do males during maintenance periods (Flores, Uribe, Swalve, & O'Dell, 2017; Grebenstein, Burroughs, Zhang, & LeSage, 2013), and that females take less time than males to begin self-administration within a session (Donny et al., 2000). Sex differences in nicotine self-administration were blocked by OVX and recovered by exogenous estradiol administration (Flores et al., 2016), indicating that sex differences in

circulating estradiol concentrations may contribute to sex differences in nicotine self-administration.

There are no sex differences in locomotor activity after a single injection of nicotine (Booze et al., 1999; Harrod et al., 2004; Kuo et al., 1999; McCormick et al., 2004), however, females demonstrate greater sensitization of locomotor activity than do males to repeated nicotine exposures (Booze et al., 1999; Harrod et al., 2004; Kanýt et al., 1999). Female rats that have been sensitized to nicotine also exhibit a greater locomotor response to a challenge of amphetamine injection than do males sensitized with nicotine (McCormick, Robarts, Kopeikina, & Kelsey, 2005). In vivo micro-dialysis experiments indicate that estradiol replacement enhances dopamine release in the NAcc after nicotine injection in OVX female (but not in castrated male) rats (Dluzen & Anderson, 1997). OVX does not alter the locomotor response to the first nicotine administration, but has been reported to attenuate the development of locomotor sensitization to repeated nicotine injections (Kanýt et al., 1999; Kuo et al., 1999). Estradiol also reversed the dampening effect of OVX on the development of locomotor sensitization to 21 daily nicotine injections (Kanýt et al., 1999). Taken together, these findings indicate that estradiol may specifically enhance sensitization in females, without producing measurable differences during the initial drug exposure.

Reports that have aimed to separate the induction and expression phases of nicotine sensitization to date have primarily focused on models that involve male subjects (Gao et al., 2014; Goutier et al., 2016; Shim et al., 2001). Thus, it remains unknown whether the estradiol's faciliatory effects on sensitization in females occurs during the induction of and / or the expression of sensitization.

Evidence for a role of membrane estrogen receptors in enhancing effects on drug-seeking behaviour

When OVX rats were treated with estradiol or both estradiol and tamoxifen, there was no significant difference in amphetamine-induced dopamine release, and both groups demonstrated greater dopamine release than untreated OVX females (Becker, James & Mermelstein, 1996). Because tamoxifen did not block the efficacy of estradiol to enhance dopamine release after amphetamine injection, the authors concluded that the enhancing effects of estradiol could be acting through g-coupled protein estrogen receptors (ex. GPER1), rather than the canonical intracellular units (ERα / ERß).

One study investigated the effects of a 12-day tamoxifen pre-treatment on the development of conditioned place preference for nicotine, and reported that tamoxifen enhanced preference for the nicotine-paired compartment (Yararbas & Pogun, 2011). Because freely cycling rats were used in this experiment, it is unclear whether enhancement of preference for the nicotine-paired environment was driven by activation of GPER1 or silencing of ERα / ERß.

Research Goals of the Current Series of Experiments

Nicotine remains under-researched relative to other psychostimulants despite its significant health consequences to humans, and animal models involving females are especially lacking. The report from Kanýt et al. (1999) is the only available peer-reviewed paper investigating the effects of estradiol treatment in OVX female rats during sensitization of locomotor activity to nicotine. In that study, 21 daily injections of nicotine with or without daily co-administration of estradiol, and locomotor activity was recorded in the hour after injection on each day. Estradiol treated rats demonstrated greater sensitization than OVX rats not receiving estradiol, and the difference between the OVX and the OVX + estradiol groups increased across

days of testing. Between-group comparisons of distance travelled on each day of testing indicated that OVX + estradiol rats travelled longer distances than the control group beginning on test day 18, and that this difference remained significant to test day 21. Because nicotine was administered repeatedly for many days in Kanýt et al. (1999), it is unclear whether estradiol enhanced the induction phase and / or the expression phase of nicotine sensitization.

Thus, the purpose of the current experiments was to investigate the role of estradiol during the induction and the expression of nicotine-induced locomotor sensitization in female rats. I first investigated whether a 2-induction protocol would produce sensitization for nicotine as it did for amphetamine (Zovkic & McCormick 2019) in naturally cycling females. Next, I investigated the role of estradiol injection selectively during the induction and / or the expression of nicotine sensitization in an OVX model. Based upon findings from these experiments, I injected tamoxifen rather than estradiol during nicotine sensitization to investigate whether estradiol's faciliatory effects depend on membrane bound estradiol receptors (e.g., GPER1) in both gonadally intact and females OVX. Lastly, I extended the induction period to three injections rather than two, to separate unique effects during induction and expression from response to the third nicotine, and investigated effects of estradiol and tamoxifen during on nicotine sensitization in OVX rats.

Expt.	Rat Status	Induction Treatment (2X)	Challenge Treatment (1X)	# of Groups	Rats / Group	Research Question
1	Intact	SAL	NIC	2	16	Does Nicotine Produce Sensitization in a 2-induction paradigm in freely cycling females?
		NIC	NIC			
2	OVX	NIC + OIL	NIC + OIL	4	12	Does OVX / injection of estradiol-benzoate alter the induction and / or the expression of nicotine sensitization?
		NIC + OIL	NIC + EB			
		NIC + EB	NIC + OIL			
		NIC + EB	NIC + EB			
3A	OVX	NIC + OIL	NIC + OIL	4	12	Will enhancement of sensitization also be visible with more limited estradiol exposure than administered in experiment 2?
		NIC + OIL	NIC + E2			
		NIC + E2	NIC + OIL			
		NIC + E2	NIC + E2			
3B	OVX	SAL + OIL	NIC + OIL	4	6	Are enhancing effects of E2 during induction a result of interactions with nicotine, or pre-exposure to E2?
		SAL + OIL	NIC + E2			
		SAL + E2	NIC + OIL			
		SAL + E2	NIC + E2			
3C	OVX	SAL + OIL	SAL + OIL	2	12	Does E2 alter distance travelled across five days of testing?
		SAL + E2	SAL + E2			
4A	Intact	NIC + OIL	NIC	2	12	Is sensitization in gonadally intact females dependent on ERα / ERß?
		NIC + TAM	NIC			
4B	OVX	NIC + OIL	NIC	3	12	Is TAM sufficient to restore sensitization in gonadally intact females?
		NIC + E2	NIC			
		NIC + TAM	NIC			
4C	Intact	SAL + OIL	SAL, NIC	2	6	Does TAM alter distance travelled in the absence of nicotine?
		SAL + TAM	SAL, NIC			
5	OVX	NIC + OIL	NIC	3	12	Are the Enhancing effects of E2 / TAM specific to expression of sensitization?
		NIC + E2	NIC			
		NIC + TAM	NIC			

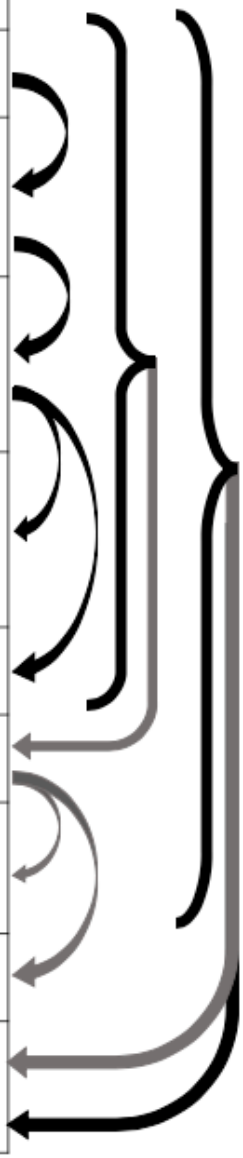

Table 1. Breakdown of experiments contained in this thesis and associated research questions. Arrows on the right-hand side of the table indicate which findings prompted the development of each subsequent research question. Black arrows indicate questions related to estradiol, and grey arrows indicate questions involving tamoxifen. See fig 2 for the injection protocol used in all experiments.

General Methods

This section contains the methods that are relevant across experiments. Separate method sections providing details that are specific to each experiment are provided later.

Rats

These experiments involved 284 female Long-Evans rats, which were purchased from Charles River (Kingston, ON) and arrived at the Brock Comparative Bioscience Facility as adults (post-natal day 55-75; rats weighed between 200 g and 350 g at the onset of testing). Rats were housed in pairs under a 12:12 hour light / dark cycle (lights on at nine am) with ad libitum access to food and water. Upon arrival at Brock University, rats were left undisturbed for a 5-day period to acclimate to the vivarium. Use of rats in these experiments was approved by the Brock Animal Care Committee, and all procedures were carried out in accordance with the Canadian Council of Animal Care guidelines.

Ovariectomy (OVX)

Immediately before surgery, rats were deeply anesthetized with inhalant isoflurane (5% for induction, 2-3% for maintenance; Sigma Aldrich). Bilateral flank surgical sites were shaved and prepped first with 7% iodine soap, then with isopropyl alcohol, and finally with 10% iodine scrub. Rats were injected subcutaneously (S.C.) with 2 mg / kg Metacam analgesic (Boehringer Ingelheim) and 5 mg / kg Baytril antibiotic (Bayer Inc.) before surgery. Baytril was diluted with approximately 800 µl of 0.9% NaCl to serve as a source of fluid replacement during recovery from OVX. Bilateral incisions were made to the flank, through which the ovaries were extracted, clamped, ligated, and removed. The abdominal muscle wall was sutured with silk thread, and the skin incision was closed using surgical staples. Rats recovered from anesthesia alone in a

designated cage for 5 minutes before being returned to their cage partners, and received 2 mg / kg Metacam (for post-operative pain management) for two days after surgery. Behavioural testing began between 5 and 10 days after OVX in all experiments.

Drugs

Nicotine. Nicotine bitartrate (NIC; Sigma Aldrich) was mixed in distilled water at a dilution of 0.4 mg / ml (nicotine calculated as the base, pH 7). Rats were injected subcutaneously (S.C.) with 0.4 mg / kg nicotine (mid-range dose known to produce sensitization in female rats; reviewed in Matta et al. 2007). 200 µl of 0.9% saline was used as a control comparison, to account for influences of injections and handling. Nicotine or saline was always administered immediately before behavioural testing.

17-ß-estradiol (E2; Sigma Aldrich**).** E2 (5 or 10 µg; see experimental procedures) was suspended in 200 µl OIL and injected S.C. 30 minutes before nicotine and behavioural testing. 200 µl of sesame oil was used as the vehicle comparison.

Estradiol-benzoate. (EB; Sigma Aldrich) in experiment 2, 5 µg EB suspended in 200 µl OIL or 200 µl of sesame OIL vehicle was and injected S.C. 30 minutes before nicotine injection and locomotor testing.

Tamoxifen. (TAM; Sigma Aldrich) In experiments 4 and 5, TAM (1 mg / kg) was suspended in sesame oil and injected S.C.. To allow for the bio-transformation of TAM to its metabolite 4-H-TAM (which has estrogenic actions whereas TAM does not; rev in Prossnitz, Arterburn & Sklar, 2007), TAM was administered 3 hours before nicotine on each induction day. 200 µl of sesame oil injected three hours before nicotine was used as the vehicle comparison.

Behavioural testing

Apparatus. The behavioural testing arenas were four 58 cm x 58 cm X 58 cm white melamine boxes; rats were tested in batches of four, in one hour test sessions under red light. Locomotor activity (distance travelled) during the hour was recorded by an overhead camera and quantified using SMART tracking software (Panlab). Arenas were cleaned with a 70% ethanol solution between batches of rats, to minimize exposure to the previous rats' scents.

Testing protocol. In all experiments, the first test session was a habituation day in which no treatment was given. Distance travelled on habituation day was used to construct treatment groups that travelled equal distances at baseline. On induction days and challenge day, rats were injected with SAL or NIC immediately before the 1 hr test session.

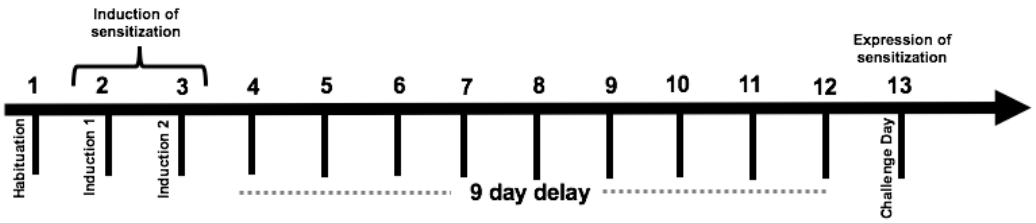

Figure 2. Injection protocol used across experiments. Rats were placed in the test arenas for 1 hour on habituation day, both induction days, and challenge day. Injections were administered on both induction days and on challenge day, after which rats were immediately placed in the testing apparatus.

Statistical analyses

SPSS version 25 was used for all statistical analyses. Mixed model ANOVAs with appropriate follow up planned contrasts and post-hoc comparisons were used to analyze distance travelled across days of testing. In circumstances where a significant main effect was obviated by a significant interaction, the main effect was not reported. In analyses with only 2 groups, independent samples t-tests were used to evaluate between-group effects, and paired-samples t-tests were used to evaluate changes across days between groups.

Experiment 1: 2-Induction Nicotine Sensitization in Gonadally-Intact Females

Introduction

The purpose of this experiment was to investigate whether the protocol that resulted in sensitization to amphetamine in females (two injections of drug, 24 h apart (induction phase) + 1 injection nine days later (expression phase); Zovkic & McCormick 2019) would produce significant nicotine-induced locomotor sensitization in gonadally-intact female rats. The prediction for this experiment was that rats would show more locomotor activity to the third injection (expression phase) of nicotine than to the second (induction phase), and that they would show more locomotor activity relative to rats receiving a first injection of nicotine after treatment with saline only during the induction phase.

Methods

Female rats were injected with either 0.4 mg / kg nicotine (NIC) or 1 mg / ml saline (SAL), and immediately placed in the test arena for 1 hr on two consecutive induction days (2 groups; n = 16 / group). Nine days later, both groups were injected with 0.4 mg / kg NIC to investigate sensitization.

Results

Body Weight. To investigate whether nicotine altered weight gain across the experiment, a repeated measures ANOVA was calculated on habituation weight and challenge day weight with drug treatment (NIC or SAL) as a between subjects variable. This analysis indicated that rats gained weight between habituation day and challenge day (F (1,26) = 33.19, $p < 0.001$), that there was no main effect of drug treatment (F (1,26) = 1.41, $p = 0.25$), and that drug treatment did not interact with weight gain (F (1,26) = 1.46, $p = 0.24$). These results indicate that two injections of nicotine did not alter the amount of weight gain across the next 9 days (Fig. 3A).

Distance travelled. To investigate effects of nicotine injections on distance travelled during the induction period, a repeated measures ANOVA was calculated on distance travelled on induction 1 and induction 2 with drug treatment (SAL or NIC) as the between groups factor. The interaction between day and drug was significant (F (1,26) = 26.18, p <.001), and follow up pairwise comparisons indicated that rats in the NIC group increased the distance travelled from the first to the second induction day ($p < 0.001$), whereas SAL-injected control rats did not differ in distance travelled on induction 1 and induction 2 ($p = 0.23$; see Fig. 3B). Additionally, SAL and NIC treated rats did not differ in distance travelled on induction 1 ($p = 0.99$), and the NIC treated group travelled longer distances than the SAL group on induction 2 ($p < 0.001$).

As predicted, on the challenge day (when all rats were injected with nicotine), NIC-pre-treated rats travelled longer distances than did SAL-pretreated controls (t (30)= 2.96, $p = 0.006$; Fig. 3B), indicating that two induction nicotine injections produce sensitization based on a between-groups comparison. Additionally, the average distance travelled in the NIC-pretreated group was higher on challenge day than on induction 2 (paired t (14) = 2.87, $p = 0.012$), indicating that 2 induction nicotine injections also produce to a nicotine challenge nine days later based on a within-groups comparison (Fig. 3C). Although SAL pre-treated rats also travelled longer distances on challenge day than on induction 2 (Fig. 3C), challenge day distance among NIC pre-treated rats was significantly higher than their baseline distance travelled on habituation day (1 sample t-test vs 100% t (15) = 5.02, p <.001), whereas challenge day distance was not different from habituation distance travelled for SAL pre-treated controls (t (15) = 0.86, $p = 0.40$ Fig. 3D).

Discussion

Two induction injections of nicotine were sufficient to produce locomotor sensitization to a challenge nicotine injection nine days after the induction period. Results from this experiment indicate that the 2-induction paradigm is sufficient to produce statistically significant locomotor sensitization to nicotine on challenge day as measured by a variety of analysis techniques: (1) Nicotine pre-treated rats increased distance travelled from induction 1 to induction 2 whereas saline pre-treated rats did not. (2) between groups, nicotine treated rats travelled longer distances than did saline treated controls on induction 2 and challenge day. (3) Nicotine pre-treated rats travelled longer distances on challenge day than induction 2. (4) Challenge day distance was significantly greater than habituation distance for nicotine-treated rats only. Thus, the 2-induction paradigm was sufficient to produce sensitization to nicotine administered nine days later in female rats.

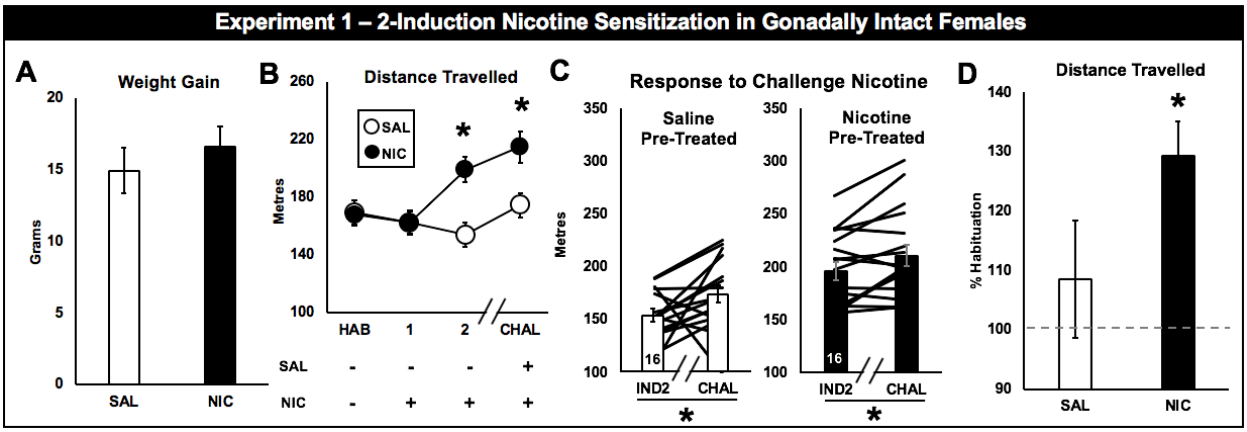

Figure 3. Means + / - S.E.M. (A) Nicotine administration during induction did not alter weight gain across the 13-day study. (B) Distance travelled on each of the 4 test days: The nicotine pre-treated group travelled longer distances on induction 2 and challenge day (C) Both saline- and nicotine- pre-treated rats travelled longer distances on challenge than on induction 2, however, (D) shows that challenge day distance is only greater than baseline distance travelled for nicotine

pre-treated rats. The numbers on the left hand bars in (C) indicate number of rats per group, * indicates $p < 0.05$.

Experiment 2: Effects of OVX and EB on Induction and Expression of Nicotine Sensitization

Introduction

Given that gonadally intact females demonstrated nicotine sensitization after 2 induction injections in experiment 1, the contribution of estradiol during the induction and / or the expression phase of sensitization was investigated. The purpose of this experiment was to test whether estradiol enhances the induction and / or the expression of locomotor sensitization to nicotine in OVX female rats, relative to untreated OVX females. Based on our lab's previous findings with amphetamine (Zovkic & McCormick 2019), the prediction for this experiment was that estradiol on challenge day, irrespective of treatment during the induction phase, would enhance the expression of locomotor sensitization.

Methods

OVX female rats were administered either 5 µg EB or 200 µl sesame oil vehicle (OIL) 30 min before each induction session. Half of each of these two groups was administered EB 30 min before testing on challenge day, and the rest were administered OIL such that half the rats received the same treatment during induction and on challenge day, whereas the rest received opposite treatments (2 X 2 design, n = 11-12 per group). Estradiol treatment is known to attenuate weight-gain in OVX female rats (Asarian & Geary, 2002), therefore, we used weight gained across the experiment to confirm that EB had been administered correctly during the induction phase.

Results

Body Weight. On the challenge day, rats that were injected with EB during the induction period weighed significantly less than did rats that were injected with OIL (t (1,45) = 3.85 $p <$

0.001). Additionally, rats treated with EB during induction also gained less weight between habituation and challenge than did OIL pre-treated OVX females (t (1,45) = 8.99, $p < 0.001$, Fig. 4A). Reduced weight gain in OVX rats treated with EB confirms estrogenic actions during the induction phase.

Distance travelled. A repeated measures ANOVA on distance travelled on induction 1 and induction 2 with induction hormone (OIL or EB) entered as the between subjects factor indicated that rats travelled longer distances on induction 2 than on induction 1 (F (1,45) = 42.45, $p < 0.001$, Fig. 4B). The between groups effect of induction hormone (F (1,45) = 4.04, p = 0.053, see Fig. 4B), and the interaction of induction hormone and day (F (1,45) = 0.34, p = 0.56) were non-significant.

To evaluate the expression of sensitization, a repeated measures ANOVA was calculated on distance travelled on induction 2 and distance travelled on challenge day, with induction hormone (OIL or EB) and challenge hormone (OIL or EB) entered as the between subject variables. This model indicated a significant day by induction hormone interaction (F (1,43) = 12.64, p = 0.001), and follow up pairwise comparisons indicated that EB pretreated rats increased the distance travelled between induction 2 and challenge day ($p < 0.001$), whereas the OIL pretreated controls did not (p = 0.25, Fig. 4C). There was no group difference in distance travelled on induction 2 (p = 0.054), however, on the challenge day, rats that were treated with EB during induction travelled significantly longer distances than rats that were treated with OIL ($p < 0.001$). The interaction between day and challenge hormone (p = 0.08), and the three-way interaction between day, induction hormone, and challenge hormone (p = 0.86) were not significant.

Discussion

Contrary to the prediction that EB would enhance the expression of nicotine sensitization on challenge day, EB during the induction phase of nicotine sensitization enhanced the expression of sensitization nine days later, and that this enhancement did not depend on the presence of EB during expression. Different effects of EB on amphetamine vs nicotine sensitization may reflect the different pharmacodynamics of each drug.

The results also indicate that OVX attenuates the expression of sensitization to nicotine, as OVX rats did not increase the distance travelled between the second induction day and challenge day. In combination with findings from experiment 1, the results suggest that estradiol during the induction phase is enabling nicotine sensitization rather than enhancing it, given that freely cycling female rats (experiment 1) also express sensitization on the challenge day.

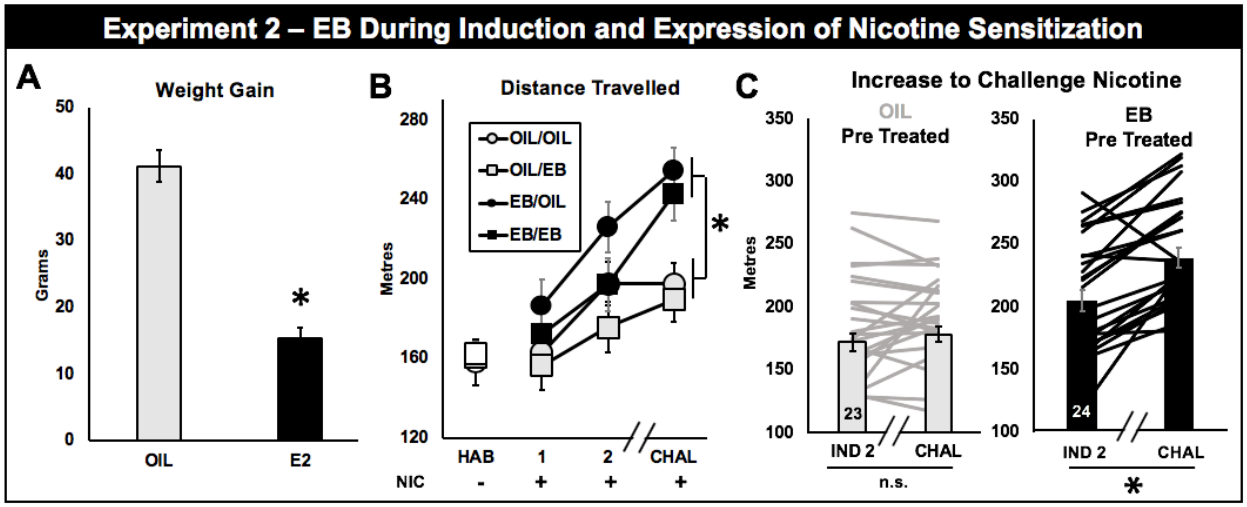

Figure 4. Means + / - S.E.M. (A) EB during induction attenuated weight gain across the 13-day experiment. (B) EB pre-treated rats (filled shapes) travelled longer distances than did OIL pre-treated controls on challenge day (open shapes). (C) OIL pre-treated rats did not increase distance travelled from induction 2 to challenge day; EB pre-treated rats travelled longer distances on challenge day than on induction 2. *Indicates $p < 0.05$.

Experiment 3A,B,C: Effects of OVX and E2 on Induction and Expression of Nicotine Sensitization

Because only rats treated with EB during the induction phase exhibited sensitization on the challenge day in experiment 2, 3 follow-up studies (3A, 3B, 3C) were conducted to investigate whether the effect reported in experiment 2 is specific to the induction of nicotine sensitization. Rats in this experiment were testing for locomotor activity in the absence of nicotine the day after challenge day, to investigate whether estradiol's effects on locomotor activity carry over to a drug-free state. There were three primary aims for this series of follow up experiments. The aim of experiment 3A was to investigate whether 17-ß-estradiol (E2) would produce the same enhancement of nicotine sensitization that was found with estradiol-benzoate (EB; experiment 2). Whereas E2 clears the system within 12 hours, whereas estradiol-benzoate has a minimum of 2x the duration of action (Leyendecker et al. 1975). Therefore, it is possible that in experiment 2, EB from induction 1 was still circulating 24 hours later when the second EB injection was given in experiment 2, meaning that EB could have accumulated beyond the intended dose and remained circulating for several days after induction 2. Thus, E2 rather than EB was administered in this experiment to limit exposure to estradiol to the time of nicotine injection and locomotor testing.

The second aim was to investigate whether two injections of estradiol produces enhanced locomotor response to a nicotine injection nine days later in drug-naïve rats (experiment 3B). The third aim was to investigate whether estradiol alters locomotor activity in the absence of nicotine (experiment 3C).

Because estradiol treatment is known to attenuate weight gain in OVX female rats (Asarian & Geary, 2002), weight gain across the experiment was monitored, and weight changes

between rats treated with estradiol and vs vehicle were compared to confirm estrogenic actions during the induction phase.

Experiment 3A Methods

As in experiment 2, OVX female rats were administered E2 during the induction and / or the expression of nicotine sensitization. 24 rats were injected with E2 30 minutes before nicotine on both induction days, and the other 24 were injected with OIL. On challenge day, half of each group was injected with E2 30 minutes before nicotine and locomotor testing (2 X 2 design, n = 12 / group). The day after challenge day, rats were injected with saline to determine whether the previous nicotine and / or estradiol altered distance travelled in the absence of nicotine.

Results. A repeated measures ANOVA to assess the effect of E2 on locomotor activity during the induction phase indicated that rats travelled longer distances on induction 2 than on induction 1 (F (1,44) = 26.27, $p < 0.001$), that there was no significant between-groups effect of treatment (OIL or E2; F (1,44) = 3.90, $p = 0.051$), and that treatment did not interact induction day (F (1,44) = 1.20, $p = 0.28$, Fig. 5A).

to investigate the expression of sensitization, an ANOVA was computed on distance travelled on induction 2 and on challenge day. Similar to the results from experiment 2, this analysis found that induction hormone interacted with test day (F (1,40) = 4.12, $p = 0.048$). Follow up pairwise comparisons of the significant interaction indicated that rats treated with OIL or E2 during induction did not differ in distance travelled on induction 2 ($p = 0.051$, see Fig. 5A), or on the challenge day ($F = 0.41$, $p = 0.52$). Nevertheless, rats treated with E2 during the induction phase travelled longer distances on challenge day than on induction 2 ($p = 0.001$, Fig. 5B), whereas OIL pre-treated rats did not ($p = 0.57$, Fig. 5B).

The effect of challenge day hormone (F (1,40) = 1.04, p = 0.31), the interaction of challenge hormone and day (F (1,40) = 0.54, p = 0.47), and the three-way interaction between day, induction hormone and challenge hormone (F (1,40) = 0.88, p = 0.35) were non-significant.

The day after challenge day when all rats were injected with saline, there were no main effects or interactions with hormone treatment at either timepoint (all p's > 0.66), indicating that the enhancing effects of estradiol during induction on expression of nicotine sensitization does not carry over to a drug-free state.

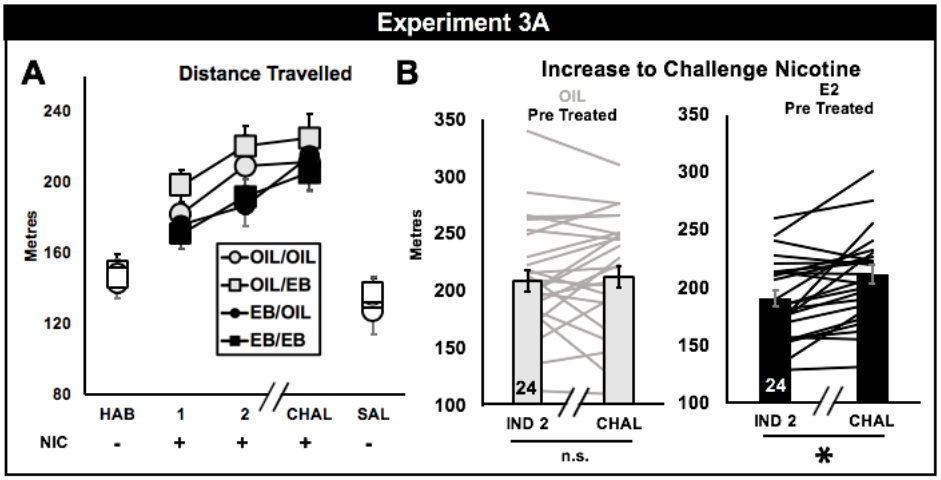

Figure 5. Mean + / - S.E.M. (A) Group means for distance travelled on each of the 5 test days. (B) only E2 pre-treated rats travelled longer distances on challenge day as compared to induction 2. * indicates $p < 0.05$.

Experiment 3B Methods

To investigate whether estradiol enhances locomotor activity to nicotine in the absence of nicotine injections during induction, OVX female rats were injected with saline (with or without E2) during the induction phase, and were administered nicotine (with or without E2) for the first time 9 days later (2 x 2 design as described above, 4 groups, n = 6 per group). The day after

challenge day, rats were injected with saline to determine whether the previous nicotine and / or estradiol altered general locomotor activity.

Results. A repeated measures ANOVA on the effect of E2 during the induction phase indicated that there was no main effect of day (F (1,22) = 0.79, p = 0.38) and no interaction of day and hormone (F (1,22) = 0.36, p = 0.55).

An ANOVA on the effect of E2 during induction and / or challenge day indicated that rats travelled longer distances on challenge day than on induction 2 (F (1,20) = 23.80, p <0.001), and that there was no significant main effect or interaction with estradiol treatment at either timepoint (all p's >.05). Although rats did travel longer distances in response to first nicotine than they had on induction 2, the average distance travelled on challenge day was not different than average distance travelled on habituation day (F (1,22) = 0.19, p = 0.67, see Fig. 6).

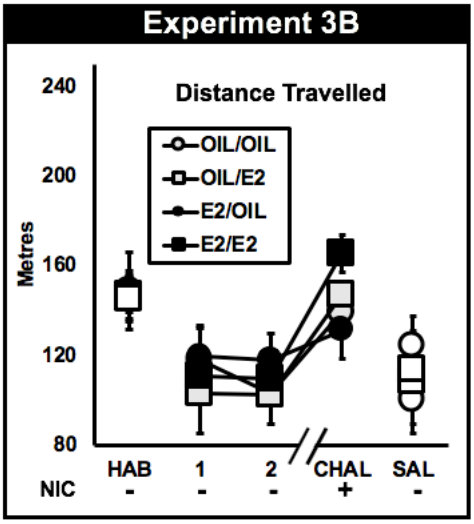

Figure 6. Means + / - S.E.M. There was no effect of estradiol treatment during induction or on challenge day, on first response to a challenge nicotine injection in drug-naïve OVX female rats.

Experiment 3C Methods

To investigate whether estradiol produced any effects on distance travelled in the absence of nicotine, estradiol was repeatedly administered during testing for locomotor activity in the

absence of nicotine. Half the rats were injected with 10 µg E2 on both induction days and challenge day; the remaining rats were injected with OIL on all three test dates (2 groups, n = 12 / group). This experiment tested the possibility that E2 enhances general locomotor activity on challenge day in the absence of nicotine. The day after challenge day, rats were injected with saline (no hormones) to determine whether previous exposure to estradiol altered distance travelled.

Results. Because rats in this study were never injected with nicotine, a repeated measures ANOVA was computed on distance travelled on all 5 days of testing with hormone treatment (OIL or E2) as the between subjects factor. Rats demonstrated a decline in locomotor activity across days of testing (F (4,22) = 15.29, p = 0.001, Fig. 7). There was no main effect of E2 treatment on distance travelled across the days of testing (F (4,22) = 0.54, p = 0.47), and E2 administration did not interact with locomotor activity across days (F (4,22) = 1.61, p = 0.21, see Fig. 7).

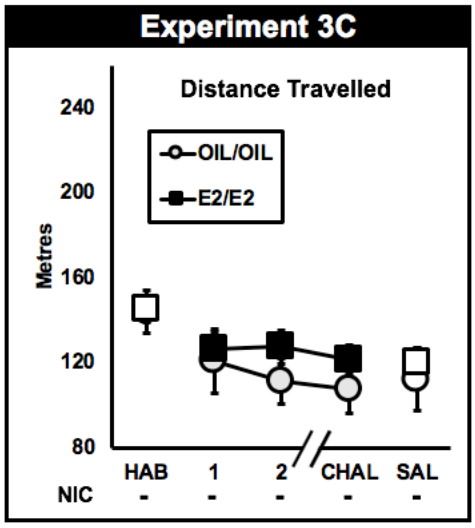

Figure 7. Means + / - S.E.M. Estradiol treatment did not alter general locomotor activity (without nicotine) across days of testing.

Body Weight (Experiments 3A, B and C Combined)

Repeated measures ANOVA on weight gained between habituation and challenge day indicated that rats gained weight across the experiment (F (1,94) = 695.13, $p < 0.001$), and that study group (A B or C) did not interact with weight gain (F (1,94) = 1.67, $p = 0.19$). Because there was no effect of study group on change in weight across the experiment, the effects of E2 during induction across all nicotine conditions were compared in order to maximize power. Similar to experiment 2, rats that were administered E2 during induction gained less weight than did OIL pre-treated controls across the experiment (t (1,94) = 2.85, $p = 0.005$), which is in keeping with previous reports of estradiol's attenuating effects on weight gain in OVX females.

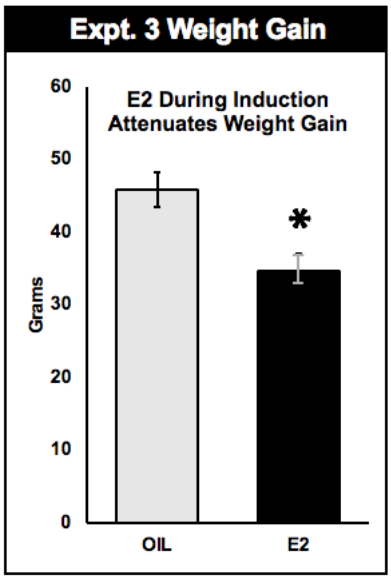

Figure 8. Mean weight gain across the experiment + / - S.E.M. Rats treated with estradiol during the induction phase gained significantly less weight between habituation and challenge day than oil-treated controls. Collapsed across all nicotine conditions (n = 48 / group).

Discussion

In both the current experiment and experiment 2, OVX female rats that did not receive estradiol injections during induction did not increase distance travelled between induction 2 and challenge day, indicating that estradiol during induction may be necessary to produce expression

of sensitization nine days later. Taken together, Experiments 1, 2 & 3A indicate that gonadally intact female rats show sensitization when challenged with nicotine after a 9-day delay, that OVX attenuates sensitization, which can be prevented by E2 limited to the induction phase. Results from experiments 3B and 3C indicate that the enhancing effects of estradiol on locomotor activity are specific to nicotine-sensitization, as E2 did not produce differences in response to first nicotine nine days later (experiment 3B), or the response to a saline injection 9 days later (experiment 3C). Thus, estradiol appears to facilitate the expression of sensitization, as expression of sensitization can be blocked by depletion of E2 (via OVX)

Experiment 4A: Tamoxifen During Induction of Nicotine Sensitization in Gonadally Intact Female Rats

Introduction

Because experiments 2 and 3 indicated that estradiol during the induction of nicotine sensitization was sufficient to facilitate expression of sensitization on challenge day, the purpose of experiment 4A was to investigate receptor targets for estradiol's effects on acquisition of nicotine sensitization. Establishing the receptors that mediates the effects of estradiol on nicotine sensitization could help formulate mechanistic hypotheses to explain the effects of estradiol. In this experiment, the selective estrogen receptor modulator tamoxifen (agonist at GPER1, antagonist at ERα/ ERß see Introduction) was administered during the induction phase of nicotine sensitization. This experiment was exploratory in nature; the hypotheses was that if the enhancing effects of estradiol during induction in experiments 2 and 3A were mediated by nuclear estrogen receptors (ERα / ERß), then the administration of the tamoxifen during induction should prevent expression of sensitization in gonadally intact females. Conversely, if the enhancing effects of estradiol reported in experiments 2 and 3A were mediated by signalling at membrane estrogen receptors (e.g., GPER1), then tamoxifen should produce no effect (or possibly enhance) expression of sensitization. Thus, we administered tamoxifen only during the induction phase of nicotine sensitization in freely cycling females and investigated expression of sensitization nine days later.

Methods

Naturally cycling female rats were injected with 1 mg / kg tamoxifen (TAM; dose based on Lien, So, & Ueland, 1991) or 1 ml / kg sesame oil vehicle (OIL) 3 hrs before NIC injection on two induction days (2 groups, n = 12 / group). This timepoint was chosen to allow time to

facilitate bio-transformation of tamoxifen to its active metabolite (4-H-TAM) which has estrogenic actions. Because experiments 2 and 3 found no effect of circulating estradiol on challenge day on expression of sensitization, no treatment was administered before the challenge nicotine injection in this experiment. All rats were then injected with saline and completed a fifth 1hr open field task the day after challenge day, to evaluate effects of previous TAM on general locomotor activity.

Results

Body Weight. To assess effects of TAM treatment on weight gain, a repeated measures ANOVA on habituation weight and challenge weight was computed, with induction treatment (TAM or OIL) entered as the between subjects factor. Administration of TAM during induction interacted with weight gain (F (1,22) = 17.34, $p < 0.001$), and follow up analyses indicated that OIL treated rats gained weight between habituation and challenge day ($p < 0.001$), whereas TAM injected rats did not ($p = 0.19$, see Fig. 9A), confirming administration of TAM during induction.

Distance Travelled. A repeated measures ANOVA to evaluate effects of TAM on distance travelled found that rats travelled longer distances on the second induction day than on the first induction day (F (1,22) = 12.05 $p = 0.002$, see Fig. 9). The main effect of TAM treatment (F (1,22) = 1.605, $p = 0.22$) and the interaction between day and TAM (F (1,22) = 0.04, $p = 0.84$) were not significant, indicating that TAM did not have any effects on locomotor activity during the induction period (i.e. while it was present in circulation).

A repeated measures ANOVA on distance travelled on induction 2 and on challenge day indicated that rats travelled longer distances on challenge day than on induction 2 (F (1,22) = 44.91, $p <0.001$). The main effect of treatment (F (1,22) = 0.03, $p = 0.85$) and the interaction between day and treatment (F (1,22) = 3.71, $p = 0.067$) were non-significant.

Discussion

TAM did not have any significant effects on the induction or the expression of nicotine-induced locomotor sensitization in gonadally intact female rats. Both TAM and oil pre-treated rats demonstrated expression of sensitization on challenge day, and exposure to TAM during induction did not significantly alter the magnitude of expression (see Fig. 9D). Nevertheless, because TAM did not suppress expression of sensitization in this experiment, these results indicate that the enhancing effects of estradiol during induction do not depend on the canonical intracellular estrogen receptors ERα and ERß.

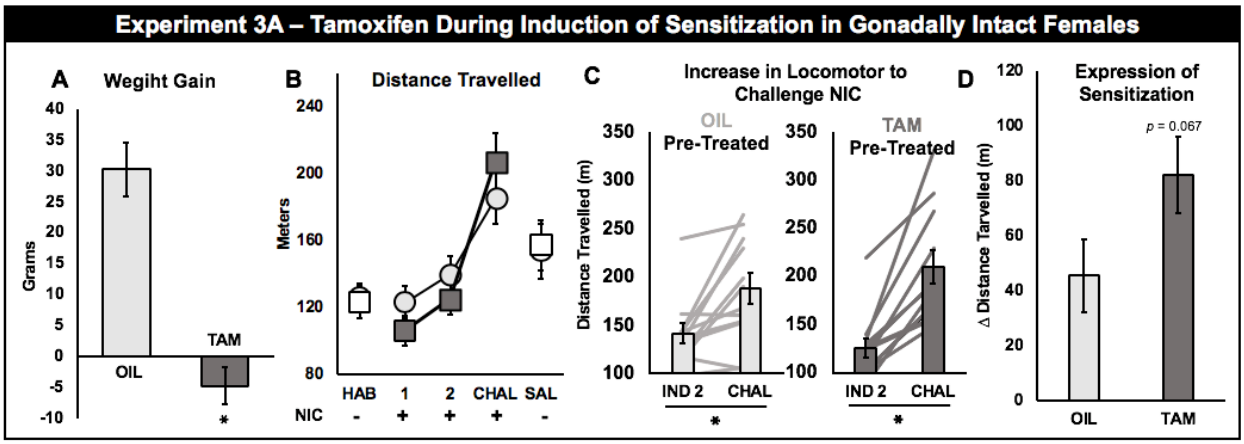

Figure 9. Means + / - S.E.M. Effects of Tamoxifen treatment on body weight and nicotine sensitization in gonadally intact females. (A) TAM pre-treated rats gained less weight than OIL treated control rats across the experiment (B) Both TAM and OIL pre-treated female rats show sensitization on challenge day (D) Distance travelled on challenge day minus distance travelled on induction 2: there was no significant effect of tamoxifen during induction on expression of sensitization.

Experiment 4B: Tamoxifen or 5 µg Estradiol During Induction of Nicotine Sensitization in OVX Rats

Introduction

Because experiment 4A indicated that TAM did not suppress expression of sensitization in gonadally intact female rats, the enhancing effects of estradiol during induction of nicotine sensitization do not depend on the canonical estrogen receptors (ERα and ERß). This result suggested that the enhancing effects may be mediated by non-traditional estrogen receptors (e.g., GPER1). Thus, the purpose of experiment 4B was to investigate the effects of the selective estrogen receptor modulator tamoxifen (TAM; agonist of GPER1, antagonist of ERα/ ERß) during the induction phase of sensitization only in OVX rats.

An estradiol-treated group was also included in this experiment, and were administered a lower dose of 17-ß-estradiol (5µg, rather than 10 µg as in experiment 3A). The purpose of this estradiol-treated group was to elucidate whether the stronger effects observed in experiment 2 as compared to experiment 3A could possibly be attributable to dose differences between experiments 2 and 3 (i.e. 5 µg estradiol-benzoate vs. 10 µg 17-ß-estradiol). Based on the previous results, the prediction for this experiment was that either TAM or estradiol during induction of nicotine sensitization would restore expression of sensitization on challenge day, and that OVX rats treated with oil vehicle during the induction phase would not express sensitization.

Methods

OVX female rats were injected either (1) 1 mg / kg TAM 3 hours before nicotine (2) 5 µg E2 30 minutes before nicotine or (3) OIL vehicle at both timepoints (3 groups, n = 8 / group). No hormone treatments were administered on the challenge day.

Results

Body Weight. Repeated measures ANOVA on weight on habituation day and challenge day indicated that weight gain across the experiment interacted with treatment (OIL, E2 or TAM; F (1,21) = 113.79, $p < 0.001$). Follow up analyses of the significant interaction indicated that there were no group differences in weight on habituation day (F (1,21) = 0.26, $p = .70$), and that weights differed as a function of hormone treatment on challenge day (F (1,21) = 8.02, $p = 0.003$). Post-hoc tests indicated that E2-treated rats gained less weight than did OVX-OIL controls (mean difference = 8 grams, $p = 0.012$), and TAM-treated rats gained less weight than did the E2-treated group (mean difference = 35 grams, $p < 0.001$, see Fig. 10A).

Distance Travelled. To investigate the effects of TAM or E2 on expression of locomotor sensitization, a repeated measures ANOVA on distance travelled on induction 2 and challenge day with induction hormone (OIL, E2 or TAM) as a between-subjects factor was computed. Results from this analysis indicated that that the increase in locomotor activity between induction 2 and challenge day interacted with treatment (F (1,21) = 7.85, $p = 0.003$, see Fig. 10B). Follow-up comparisons indicated that neither the OIL or the E2 pre-treated group travelled longer distances on challenge day than on induction 2 ($p = 0.14$; $p = 0.22$, respectively). TAM pre-treated rats, however, travelled farther on challenge day than on induction 2 ($p = 0.005$. see Fig. 10C). There were no significant group differences in distance travelled on induction 2 or on challenge day (all p's > 0.1).

Discussion

In keeping with the findings from experiment 4A wherein gonadally intact rats were administered TAM during nicotine induction, these results indicate that TAM is sufficient to enhance the expression of sensitization in OVX rats as well. In combination with findings from

experiment 4A, the results from this experiment further support the interpretation that the enhancing effects of E2 on the expression of sensitization may be mediated my membrane-bound estrogen receptors (e.g. GPER1) rather than actions involving ERα / ERß.

The finding that 5 µg E2 30 minutes before induction is not sufficient to restore sensitization in OVX rats was surprising, given that the previous results indicated that both 10 µg E2 and 5 µg EB were capable of enhancing sensitization in OVX females. Differences in the pharmacodynamics of EB, and differences in the rate of clearance between E2 and EB may explain why the group of rats treated with 5 µg E2 did not demonstrate sensitization of the locomotor response on challenge day in this experiment.

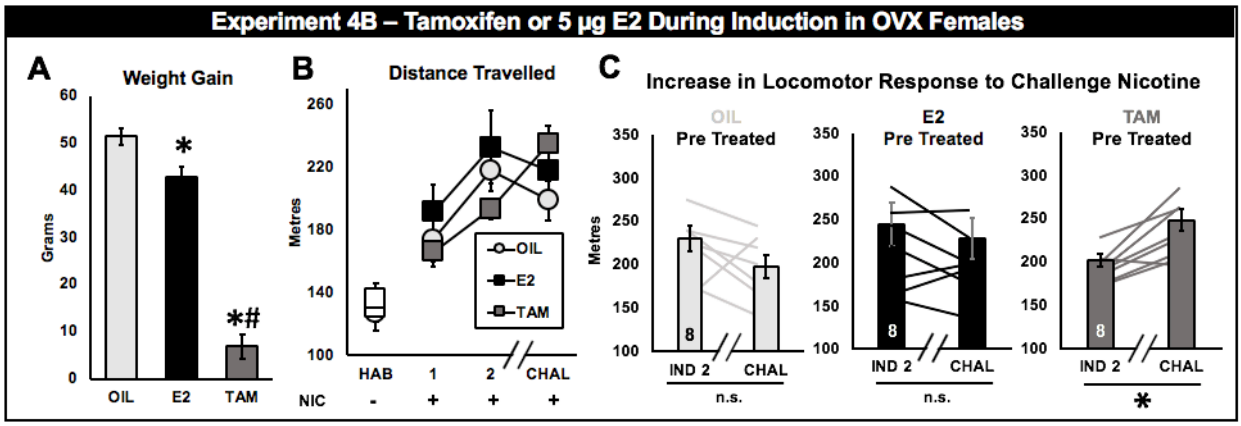

Figure 10. Means + / - S.E.M. (A) E2 or TAM attenuated weight gain across the experiment as compared to OIL-treated rats (*), and rats in the TAM group gained less weight than those in the E2 group (#). (B) All groups respond to nicotine during the induction period equally. (C) Both OIL and E2 pre-treated rats travelled equal distances on induction 2 and challenge; TAM pre-treated rats travelled longer distances on challenge day than on induction 2.

Experiment 4C: Effects of Tamoxifen Pre-Treatment on Response to Challenge Nicotine in Drug-Naïve Gonadally Intact Rats

Introduction

In experiment 4C, the effects of TAM treatment on saline-induced locomotor activity, and response to the first nicotine measured after a nine-day delay, were investigated in freely cycling female rats. The purpose of this study was to elucidate the effects of TAM nine days previously on distance travelled in response to first nicotine, to rule out the possibility that prior exposure to TAM enhanced response to a nicotine injection after a delay on its own in experiment 4B. The prediction for the experiment was that TAM would not alter general locomotor activity, or hyperlocomotion to first nicotine, based on findings from experiment 3 (which indicated that the enhancing effects of estradiol injected during the induction phase specifically enhance acquisition of nicotine sensitization, without altering saline-induced distance travelled).

Methods

Naturally cycling female rats were injected with 1mg / kg TAM (TAM) or an equal volume of the sesame oil (OIL) vehicle 3 hours before 2 saline "induction" days (2 groups, n = 6 / group). Nine days later, rats were injected with saline to investigate the effects of prior TAM treatment on general locomotor activity. The next day (10 days after TAM), rats received their first 0.4 mg / kg nicotine, to assess the influence of previous TAM exposure on response to nicotine in nicotine-naïve rats.

Results

Body Weight. Before the experiment began, there was no difference in weight between the two treatment groups (t (11) = 0.00 p = 1.0). TAM pre-treated rats tended to weigh less than

did OIL pre-treated controls on challenge day, however, this difference did not meet the threshold of statistical significance (t (11) = 2.01, p = 0.072). Across the 10-day experiment, OIL control rats' weights did not change (paired t (1,5) = 0.12, p = 0.91) whereas TAM-treated rats lost weight (paired t (1,5) = 7.72, p = 0.001, see Fig. 11A). These results are somewhat inconsistent with the other weight-data reported in this theses, largely because older rats were used in this experiment (~120 days old), which show much less weight gain relative to younger rats.

Locomotor Sensitization. Repeated measures ANOVA on distance travelled on habituation, induction 1 and Induction 2 with treatment (OIL or TAM) as the between subjects factor indicated that all rats decreased distance travelled across the induction period (F (2,10) = 4.59, p = 0.023), that there was no main effect of TAM treatment on this decrease (F (2,10) = 0.13, p = 0.73), and that there was no interaction between day and treatment (F (2,10) = 2.23, p = 0.13 see Fig. 11B). Rats increased distance travelled in response to a saline injection after a nine-day break from the testing apparatus (F (1,10) = 29.62, p < 0.001), with no main effect of TAM (F 1,10) = 0.16, p = 0.69) or interaction between day and treatment (F (1,10) = 0.76, p = 0.40). Nicotine produced significant depression of the locomotor response relative to the previous day when saline was administered (F (1,10) = 21.89, p = 0.001), however, this decrease was not significantly different than the decrease observed between Habituation and Induction 1 (F (1,10) = 1.62, p = 0.23). Three was no main effect of TAM (F (1,10) = 0.006, p = 0.94) or interaction between TAM and day (F (1,10) = 0.07, p = 0.79 see Fig. 11B).

Discussion

These findings indicate that TAM does not produce effects on saline-induced behaviour or response to first nicotine measured after a delay. Thus, the enhancing effects of TAM in

experiment 4B cannot be attributed to a direct effect of prior TAM administration. Thus, TAM appears to specifically enhance the acquisition of nicotine sensitization, because no changes in saline-induced distance travelled were observed in this experiment.

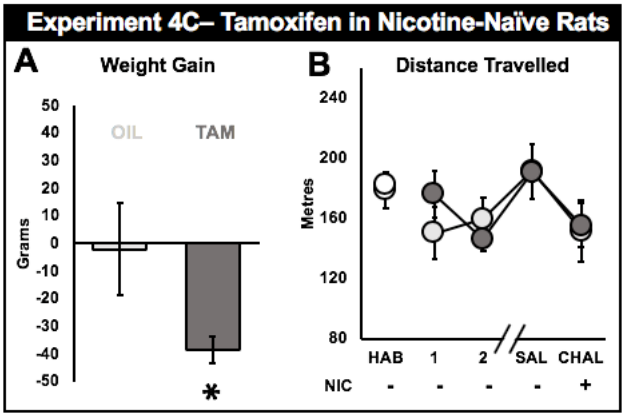

Figure 11. Means + / - S.E.M. (A) OIL treated rats did not change weight across the experiment, whereas TAM treated rats lost a significant amount of weight. (B) TAM pre-treatment did not alter response to saline or nicotine nine days later.

Experiment 5: Effects of E2 and Tamoxifen Treatment During 3 Induction Nicotine Injections in OVX Females

Introduction

Thus far, this series of experiments indicates that gonadally intact female rats increased distance travelled to challenge nicotine (experiment 1), OVX-OIL rats did not increase distance travelled from induction 2 to challenge day (experiments 2, 3, 4B), and E2 or TAM during induction can restore within-subjects sensitization. Based upon these results from experiments 1-4, the working hypothesis is that effects of E2 and TAM during induction specifically alter locomotor activity in response to nicotine during the expression phase of sensitization, which is functionally and anatomically distinct process than that involved in induction (see introduction). Based on this framework, the prediction was that all OVX rats would respond to nicotine equally during a third induction exposure (the day after induction 2), and that the effects of E2 / TAM treatment during induction would only be visible during the expression phase (i.e. after a delay). Overall, the prediction for this experiment was that estradiol treatment during nicotine induction enhances expression of sensitization specifically, rather than the response to the third nicotine.

Methods

OVX female rats were injected with 0.4 mg / kg nicotine on three consecutive induction days (an additional induction day), and challenged with a fourth injection of 0.4 mg / kg nicotine nine days later. Rats were either administered TAM (3 hours before nicotine) 5 µg E2 (30 minutes before nicotine) or sesame oil vehicle at both timepoints (3 groups, n = 12 / group). No hormone treatments were administered on challenge day.

Body Weight. A repeated measures ANOVA on habituation weight and challenge weight with treatment (OIL, E2 or TAM) entered as the between subjects variable indicated a significant

day by hormone interaction (F (2,33) = 121.98, $p < 0.001$ see Fig. 12A). Both OIL- and E2- pre-treated rats gained weight across the study (both p's < 0.001), however, TAM treated rats lost weight between habituation and challenge ($p < 0.001$, see fig 12A). Post-hoc comparisons indicated that E2 rats gained less weight than OIL rats across the experiment ($p = 0.021$), in keeping with experiments 2, 3 and 4B.

Locomotor activity. A repeated measures ANOVA on induction 1, 2 and 3 indicated that rats increased the distance travelled across the 3 induction days (F (2,33) = 80.98, $p < 0.001$). The progressive increases in locomotor activity during the three-day induction period did not interact with hormonal treatment during induction (OIL, E2 or TAM; F (2,33) = 1.05, $p = 0.39$ see Fig. 12B), and that there was no main effect of treatment on distance travelled across the 3 induction days ($F = 0.27$, $p = 0.77$). When injected with nicotine nine days later, however, hormonal pre-treatment interacted with day (F (1,33) = 21.28, $p < 0.001$). Planned contrasts for distance travelled on induction 3 vs. challenge day indicated that hormone-treated rats showed a greater expression of sensitization than did the OIL-treated control group (OIL vs TAM + E2 t (33) = 4.87, $p < 0.001$), and that TAM pre-treated rats showed a greater sensitization than did E2-pretreated rats (E2 vs TAM t (22) = 5.63, $p < 0.001$). Between group post-hoc comparisons of the difference scores between induction 3 distance and challenge distance confirmed that each differences score was unique: (OIL vs E2 $p = 0.049$, E2 vs TAM $p < 0.001$ see Fig. 12C). Follow up within-group pair-wise comparisons of distance travelled on induction 3 and challenge day indicated that OIL pre-treated rats decreased distance travelled between induction 3 and challenge day (F (1,33) = 2.89 $p = 0.019$), rats in the E2 group did not differ in distance travelled on induction 3 and challenge day (F (1,33) = 0.94, $p = .34$), and TAM treated rats increased distance travelled on challenge day relative to induction 3 (F (1,33) = 50.58, $p < 0.001$).

Discussion

Results from this experiment indicate that the effects of either E2 or TAM administered during 3 induction days produce enhanced sensitization to a challenge nicotine injection nine days later, relative to OIL injection control rats. In accordance with the hypothesis, there were no effects of E2 or TAM on distance travelled during the 3-day induction period, and, most importantly, there was no group difference in distance travelled on induction 3. Taken in combination with the previously described series of experiments involving 2 induction days, these results confirm that the effects of E2 or TAM during induction alter the response to nicotine during induction, producing group differences that may require time to emerge.

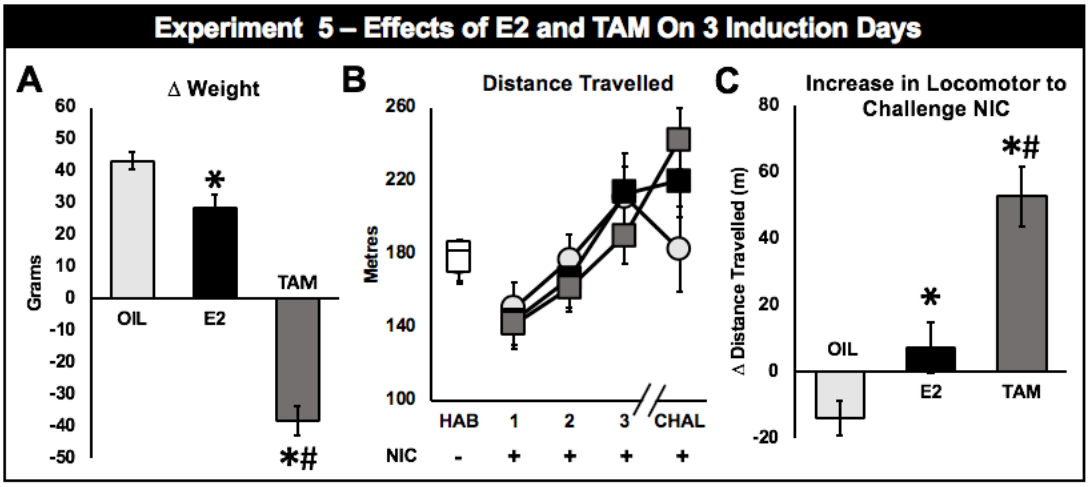

Figure 12. Means + / - S.E.M. (A) E2 during induction attenuated weight gain; TAM during induction caused weight loss. (B) All groups respond to nicotine equally during the 3 day induction period (while hormones were present in the system). (C) E2 pre-treated rats demonstrated greater expression of sensitization that OIL pre-treated controls; TAM pre-treated rats increased distance travelled significantly more than E2 treated rats (#).

Legend
No INJ
OIL
E2
TAM

Symbol	Meaning
-	No Activation
+	Activated by Treatment
✓	Activated by Endogenous E2
×	Antagonized by Treatment
↑	Statistically sig INCREASE across 9-day delay
=	No change in behaviour across 9-day delay
↓	Statistically sig DECREASE across 9-day delay

Receptor Activation During Induction and Expression of Sensitization

						Induction		Challenge		
Expt.	Gonadal Status	Rats / Group	IND Days	Replace	Dose	GPER1	ERα / ERß	GPER1	ERα / ERß	Δ Distance
1	INTACT	16	2	-	-	✓	✓	✓	✓	↑ *
2	OVX	12	2	OIL/OIL	-	-	-	-	-	=
				OIL/EB		-	-	+	+	= *
				EB/OIL	5µg	+	+	-	-	↑
				EB/EB		+	+	+	+	↑
3A	OVX	12	2	OIL/OIL	-	-	-	-	-	=
				OIL/E2		-	-	+	+	= *
				E2/OIL	10 µg	+	+	-	-	↑
				E2/E2		+	+	+	+	↑
4A	INTACT	12	2	OIL	-	✓	✓	✓	✓	↑ n.s.
				TAM	1 mg/kg	+	×	✓	✓	↑
4B	OVX	8	2	OIL	-	-	-	-	-	=
				E2	5µg	+	+	-	-	= *
				TAM	1 mg/kg	+	×	-	-	↑
5	OVX	12	3	OIL	-	-	-	-	-	↓ *
				E2	5µg	+	+	-	-	=
				TAM	1 mg/kg	+	×	-	-	↑ *

Table 2. Breakdown of estrogen receptor subtype activation in each experiment and related effects on locomotor sensitization to nicotine * indicates significant difference (p <0.05) in sensitization measured as change from the last induction day to challenge day. *Note* that only groups that were injected with nicotine on all test dates are represented in this graphic (no saline controls).

General Discussion

The purpose of these experiment was to investigate the role of estradiol during the induction and expression of nicotine sensitization in female rats. Taken together, the results indicate (1) that two injections of nicotine was sufficient to produce locomotor sensitization to a nicotine challenge nine days later (gonadally intact rats travelled longer distances on challenge day than on induction 2; experiments 1, 4A), (2) that ovariectomy (OVX) blocked the expression of sensitization (experiments 2, 3A, 4B), and (3) that administration of 10 µg estradiol (experiment 3A, (but not with 5 µg, experiment 4B)), 5 µg estradiol-benzoate (experiment 2), or tamoxifen (experiment 4B) during induction was sufficient to enable sensitization to nicotine nine days later in OVX females (see Table 1. for summary of experiments). These results indicate that the estradiol during induction facilitates nicotine sensitization via actions at membrane bound receptors (e.g., GPER1).

Estradiol during induction enabled expression of nicotine sensitization

In experiments 2 and 3A, OVX rats treated with estradiol during the induction phase travelled longer distances on the challenge day than on the second induction day. The same sensitization between induction 2 and challenge was observed in gonadally intact female rats (experiment 1, experiment 4A). Taken together, these findings indicate that circulating estradiol during nicotine induction facilitates sensitization of the locomotor response to a challenge injection of nicotine.

Estradiol did not produces enhanced locomotion when administered to OVX females in the absence of nicotine

In experiment 3C, there was no effect of estradiol on locomotor activity during five days of locomotor testing. Importantly, this finding indicates that estradiol does not enhance

locomotor activity in the absence of nicotine. Although these findings indicated no effect of estradiol on distance travelled during repeated saline injections, previous reports have found enhancing effects of estradiol on general locomotor activity in OVX females (e.g., Kanýt et al. 1999, Sell, Scalzitti, Thomas, & Cunningham, 2000). Two possible reason for this difference are the dose estradiol administered and the duration of estradiol treatment: Kanýt et al. (1999) injected OVX female rats with 50 µg / kg estradiol for 21 consecutive days, and Sell et al. 2000 implanted rats with subcutaneous slow release estrogen pellets two weeks before the onset of testing. The group difference in distance travelled between vehicle and estradiol treated rats appeared for the first time on day twelve of testing in Kanýt's (1999) paper, indicating that prolonged exposure to estradiol may be necessary to produce effects on general locomotor activity. Results from experiment 3C indicate that the estradiol injection protocol reported here was limited enough to produce no effects on general locomotor activity across five saline injections.

In experiment 3B, administration of estradiol nine days before the first nicotine injection had no effect on hyperlocomotion after nicotine. This finding indicates that the enhancing effects of estradiol observed in experiments 2 and 3A are likely not driven by the prior exposure to estradiol alone. Rather, the enhanced response to a challenge nicotine injection was observed only when estradiol and nicotine were co-administered during the induction phase of nicotine sensitization.

Possible dose-response effects of estradiol during induction of nicotine sensitization

Contrary to the prediction based on experiments 2 and 3A, administration of 5 µg of estradiol during the induction phase of nicotine sensitization did not enhance the expression of sensitization in experiment 4B. This result was surprising, as both 10 µg estradiol (experiment 3)

and 5 µg of estradiol-benzoate (experiment 2) were sufficient to restore the expression of sensitization in OVX females. It is possible that the lack of enhancing effects of 5 µg estradiol was an indication of a dose-response effect; because nuclear receptors have 2x greater affinity for estradiol than does GPER1 (Cheng et al., 2014), the lower dose administered in experiment 4B could have preferentially activated the nuclear receptors. Proportionally more binding at ERα / ERß (and less at GPER1) could contribute to a lack of effects, because the enhancing effects of estradiol on nicotine sensitization appear to involve membrane receptors specifically (discussed in a later section). The inclusion of several doses of estradiol within one study may clarify whether there are dose effects of estradiol on the induction of nicotine sensitization.

Estradiol is not required on the challenge day to facilitate the enhanced expression of nicotine sensitization

In experiments 2 and 3A, estradiol was not required on the challenge day to facilitate the enhancing effects of estradiol during the induction phase of sensitization. In fact, there was no effect of estradiol on challenge day on expression of sensitization in either experiment 2 or 3A. For this reason, hormonal treatments were not administered on the challenge day in the subsequent experiments. This finding was unexpected, because a previous report from our lab indicated that estradiol-benzoate administered 30 minutes before an amphetamine injection was sufficient to produce enhanced expression of sensitization in OVX females (Zovkic & McCormick, 2019, discussed more in a later section). The current results indicate that estradiol and nicotine interact during the induction phase of sensitization to produce an enhanced response to a subsequent nicotine challenge, with or without estradiol circulating estradiol on the challenge day. These results suggest that the brain changes that underlie the enhancing effect of

estradiol on the acquisition of nicotine sensitization likely occur during or soon after the induction phase, and are maintained for a period of nine days in the absence of estradiol.

OVX Prevents the Expression of Sensitization to Nicotine

In experiments 2, 3A, and 4B, OVX females that were administered oil vehicle during the induction phase showed no evidence of nicotine sensitization on the challenge day: There was no difference between the distance travelled on induction 2 and on challenge day (see Figs. 4 & 5). Because gonadally-intact female rats did show locomotor sensitization on the challenge day in both experiment 1 and experiment 4A, the finding that OVX rats do no express sensitization on the challenge day suggests that the depletion of ovarian hormones prevents sensitization to nicotine. Thus, gonadally-intact rats consistently expressed sensitization to a challenge nicotine injection whereas OVX females consistently did not. These results reinforce the conclusion from the results in OVX rats treated with estradiol that estradiol during induction is required for sensitization to nicotine.

OVX Females Respond to Nicotine During the Induction Phase

Although OVX female rats did not exhibit sensitization to nicotine on the challenge day, they did show an increase in distance travelled during from the first to the second induction day in experiments 2, 3A, and 4B. Thus, OVX rats do increase the locomotor response to nicotine when repeatedly administered with short intervals between injections, but they do not show the increase after a long-delay that is characteristic of sensitization. Further, in experiment 5, (which involved three induction days), OVX rats treated with oil vehicle travelled further on induction 3 than on induction 2 (see Fig. 10). There was no group difference in distance travelled on the third induction day in experiment 5, but group differences emerged 9 days later when the rats were challenged. These findings reinforce the results from the experiments that involved two

induction days, highlighting that estradiol's enhancement of sensitization is specific to the expression phase (i.e. after a delay).

These results highlight that induction and sensitization involve dissociable processes, as estradiol during the induction did not alter locomotor activity while present in the system, and only produced group differences after a delay. It may be that the molecular changes in the brain induced by nicotine diminish during the 9-day delay in OVX rats, and that estradiol or tamoxifen during induction support the nicotine-induced adaptations for the expression of sensitization to nicotine.

Involvement of GPER1 in enhanced acquisition of nicotine sensitization

Administration of tamoxifen during the induction phase of sensitization also resulted in sensitization to nicotine in OVX females for both the experiments that involved two induction days (experiment 4B) and that involving three induction days (experiment 5). The active metabolite of tamoxifen (4-H-TAM; see introduction) acts as an antagonist at the canonical intracellular estrogen receptors (ERα / ERß) while simultaneously activating the membrane estrogen receptor GPER1. In both experiments 4 and 5, OVX rats treated with tamoxifen did not differ from vehicle-treated females during the induction phase of nicotine sensitization, but only those treated with tamoxifen during induction showed sensitization to nicotine on the challenge day. These results parallel the findings in experiments 2 and 3A where estradiol was administered, suggesting that estrogenic actions – likely at GPER1 – are required during the induction phase to facilitate the expression of nicotine sensitization nine days later.

In experiment 4A, administration of tamoxifen to gonadally-intact rats during the induction phase of nicotine sensitization did not alter expression of sensitization on the challenge day; both tamoxifen-treated and vehicle-treated rats showed sensitization to nicotine. In

experiment 4A, all rats would have had endogenous circulating estradiol throughout the experiment, and both groups would have experienced activation at GPER1 during nicotine induction (see table 2). The conclusion from the experiments investigating the effects of tamoxifen on nicotine sensitization is that estradiol during induction enables expression of nicotine sensitization nine days later via actions at membrane bound estrogen receptors (e.g. GPER1). Further, these findings indicate that silencing ERα / ERß does not alter the induction or the expression of nicotine sensitization.

In experiment 4C, tamoxifen treatment in the absence of nicotine had no effect on locomotor activity. Rats treated with tamoxifen did not differ from rats given vehicle in response to a saline injection nine days later, or the first nicotine injection the next day. Thus, consistent with the findings with estradiol, co-administration of nicotine and tamoxifen during the induction phase was required to produce an enhanced locomotor response to a challenge nicotine injection.

Possible mechanisms for estradiol's effects on the expression of nicotine sensitization

Estradiol is known to enhance the development and survival of new dendritic spines in the hippocampus during memory formation and consolidation (Luine, 2016). Intermittent exposure to nicotine is also known to increase dendritic complexity in the brain, but these brain changes are typically limited to the reward pathway (Brown & Kolb, 2001; Li, Kolb, & Robinson, 2003; Mychasiuk, Muhammad, Gibb, & Kolb, 2013; Robinson & Kolb, 1999). Repeated injection of various drugs (including nicotine) induces a long-term-potentiation (LTP)-like state in ventral tegmental area (VTA) neurons, which is thought to facilitate the development of new or strengthened synapses in downstream brain regions (i.e. NAcc; Nestler, 2013). Antagonism of n-methyl-d-aspartate (NMDA) receptors has been shown to block the induction (but not the expression) of sensitization, indicating that LTP may be critical during induction to

produce sensitization (Kelsey, Beer, Lee, & Wagner, 2002; Pistillo, Clementi, Zoli, & Gotti, 2015). One possible mechanism that could explain the findings reported here is that estradiol during induction enhanced the consolidation of the LTP-dependent processes that are critical for induction of sensitization. Increased dendritic arborization within the NAcc may facilitate greater responses to drugs by allowing for more diffuse signalling and activation in response to challenge injection (rev. in Nestler 2013). It is possible that co-administration of estradiol or tamoxifen during the induction phase of sensitization augmented the increase in dendritic density in key nodes of reward processing in the brain, which could be involved in facilitating a greater locomotor response to a challenge nicotine injection.

GPER1 involvement in cognitive enhancements by estradiol: evidence from studies of learning and memory

Estradiol has a known to promote memory retention in female rodents (Frick & Kim, 2018), and estradiol has also been shown to increases dendritic arborization in the hippocampus. Depletion of ovarian hormones via OVX impairs memory retention after a learning event (Frick, 2015; Tuscher, Fortress, Kim, & Frick, 2015) and administration of estradiol before or after the learning event is sufficient to reverse OVX-induced deficits (Gresack & Frick, 2006). Estradiol bound to bovine albinum serum (which prevents estradiol from entering cells, thus blocking actions of intracellular receptors while leaving membrane receptors functioning) is also sufficient to produce memory enhancements in OVX rodents (Fernandez et al., 2008), indicating that estradiol's effects on memory in females could also be mediated by membrane bound receptors (e.g., GPER1). Stereotaxic injection of the GPER1 specific agonist G-1 to the hippocampus was also sufficient to enhance object- and social- memory, further indicating involvement of GPER

in cognitive enhancements by estradiol (Lymer, Robinson, Winters, & Choleris, 2017; Sharice et al., 2015).

Activation of GPER1 may have a key role in facilitating brain changes that are induced by learning, as its activation is thought to produce alterations in synaptic scaffolding, which could increase synaptic strength (Waters et al., 2015). Although the mechanisms involved in estradiol's enhancing effects on learning are not fully characterized, rapid increases in cellular plasticity and synaptic scaffolding, which ultimately could facilitate enhanced dendritic complexity offer a framework to explain enhanced effects of estradiol treatment in OVX females. It could be that co-administration of a GPER1 agonist and nicotine produces greater increases in dendritic arborization than nicotine alone, producing locomotor sensitization on challenge day only in groups that had circulating GPER1 agonists during the induction phase. Alternatively, activation of membrane estrogen receptors during nicotine induction could have

Enhancing effects of estradiol on nicotine sensitization vs. amphetamine sensitization

A previous report from our lab where estradiol-benzoate was administered selectively during induction and / or expression of amphetamine-induced locomotor sensitization (using the same 2-induction paradigm and OVX female rats) found that circulating estradiol on challenge day enhanced expression of amphetamine sensitization (Zovkic & McCormick, 2019). The findings reported in Zovkic and McCormick (2019) are in keeping with similar work involving amphetamine: stereotaxic estradiol injected in the dorsal striatum has been shown to enhance dopamine overflow after amphetamine 30 minutes later in vivo (Cummings et al., 2014; Madularu, Shams, & Brake, 2014), and in striatal slices (Becker & Beer, 1986). Enhancing effects of estradiol on dopamine release after amphetamine were not blocked by coadministration of tamoxifen (Mermelstein, Becker, & Surmeier, 1996), indicating that membrane receptors

might also mediate enhanced responsivity to amphetamine, albeit along a different time course than for nicotine.

Different enhancing effects of estradiol on amphetamine and nicotine sensitization may also reflect unique pharmacodynamics of each drug. Locomotor sensitization develops differently in response to repeated injections of amphetamine vs. nicotine: nicotine sensitization develops in a linear pattern across exposures, whereas amphetamine initially causes a greater increase in locomotor activity than nicotine, then produces little within-subjects change across subsequent administrations. Further, the enhancing effects of estradiol on challenge day reported in Zovkic & McCormick (2019) were analyzed as distance travelled on challenge day compared to first response to amphetamine (induction 1). In the series of experiments reported here, every group in every experiment demonstrates statistically significant locomotor sensitization between induction 1 and challenge day, which may be partially driven by initial locomotor depressant effects of nicotine (i.e. see induction 1 in experiment 1). To avoid influences of first-day suppression of locomotor activity which is sometimes (but not always, i.e. see experiments 2 & 3) produced by nicotine, challenge day distance was analyzed relative to induction 2 distance throughout the experiments reported here. Given that nicotine and amphetamine excite mesolimbic circuitry via different mechanisms, it is possible that estradiol's enhancing effects on sensitization could be specific to each drug.

The findings reported in this thesis emphasize that it may not be appropriate to extrapolate findings involving the modulatory effects of estradiol on drug responses between different drugs. Nicotine remains under-researched relative to illicit drugs, despite widespread availability and use in human populations. More research on the effects of nicotine on drug

seeking behaviours are required to fully understand how and when estradiol enhances responsivity.

Enhanced sensitization and addiction

Individual differences in the magnitude of nicotine-induced sensitization across seven injections predicted phasic dopamine release in the NAcc to the eighth injection, indicating that sensitization could be a biomarker of individual differences in susceptibility to nicotine-induced brain changes (Fennell et al., 2019). The basic findings reported here indicate that activation of GPER1 during nicotine induction modulates acquisition of sensitization during the first 2 or 3 nicotine exposures in female rats, producing measurable differences in response to a challenge nicotine after a delay. Sensitization of the incentive salience of drugs during early exposure offers a plausible framework for enhanced addiction vulnerability in females relative to males, as greater sensitization could promote the transition to from casual to chronic use, and may be a key first step the in the development of pathological addiction. The extent to which sex-specific patterns of sensitization during the first few exposures to drugs contribute to reported sex differences in addiction vulnerability remains to be explored empirically.

Future directions

Further pharmacological investigations could confirm the interpretations of the current data presented here, by using the highly selective agonist and antagonist of GPER1 (G1 & G15 respectively – Tocris Biotech). These agents cannot cross the blood-brain barrier, and therefore require stereotaxic administration via cannulation of relevant brain targets. An interesting next experiment could be to administer these G1 / G15 via cannulation to both key reward regions (ie. The NAcc, VTA), and non-reward related control regions (e.g., dorsal hippocampus), to establish regional specificity for the modulatory effects of estradiol on nicotine sensitization. Based on the

findings from the MA experiments, I predict that G1 (GPER1 agonist) administered to OVX rats only during the induction phase of nicotine sensitization would be sufficient to restore expression of sensitization to a challenge nicotine injection. Further, I predict that stereotaxic administration of G15 (GPER1 antagonist) only during induction of nicotine sensitization in gonadally intact female should be sufficient to block expression of sensitization to nicotine nine days later. I hypothesize that the actions of these agents would be specific to administration to the reward pathway; however, this hypothesis is based only on the existing literature and on speculation. Establishing brain targets for the enhancing effects of estradiol on acquisition of nicotine sensitization could aid in the development of more tailored mechanistic hypotheses about estradiol's effects in the brain during acquisition of nicotine sensitization.

More studies are needed that involve acute administration of estradiol via injection to better understand how and when estradiol enhances other reward motivated behaviours in females (e.g., self-administration, conditioned place preference). The results reported here indicate that the enhancement of sensitization by estradiol was visible nine days later, indicating long-lasting changes induces by coadministration of estradiol and nicotine as compared to nicotine alone. Given the established links between drug sensitization and propensity to self-administer drugs of abuse, it would be interesting to investigate whether the sensitization-altering effects of estradiol reported here are sufficient to promote voluntary self-administration of drugs.

Conclusions

Findings from this series of experiments add to the growing literature indicating that membrane bound estrogen receptors are involved in modulating reward function in female rats. The finding that estradiol interacted with nicotine to produce long-lasting increases in OVX females indicates that estradiol is an important modulator of responses to nicotine during the first

three exposures to nicotine. Enhancement of sensitization during early exposure to drugs may be one factor that contributes to sex differences in addiction liability in human and animal models of drug seeking, as greater sensitization is thought to facilitate acquisition of pathological addiction via enhanced drug cravings (Berridge & Robinson, 2016).

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